Environmental Biotechnology

Theory and Application

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Gareth M. Evans
and Judith C. Furlong

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University of Durham, UK and Taeus Biotech Ltd

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This book is dedicated with much love to:

The late Brother Ramon SSF, friend and mentor, and to the late Ronald and Delcie Furlong.

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John and Denise Evans.

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Foreword

by Professor Bjørn Jensen

Chairman of the European Federation of Biotechnology, Environmental Biotechnology Section and Research and Innovation Director, DHI Water and Environment.

Environmental biotechnology has an exciting future. Just the thought of having microorganisms work for you – simply by feeding them with natural substrates and having hazards turned into minerals and nature's own basic constituents – is really intriguing. Of course, we all know that it is not that simple, but nevertheless it is both the fundamental premise, and the ultimate goal, which we must bear in mind in all developments in the field.

Environmental biotechnology is not an easy subject to cover, but therefore so much the more important that it should be. For years, the environmental technologies had a little too much tail wind because of the overwhelming sympathy for green solutions. This often led to misuse and discredit of the technologies among some end users. In those years, too many studies underestimated the complexity of the task. The inevitable outcome was poor documentation, not giving enough credit to the processes involved and the degree of process control required. The reputations of these technologies were also hampered by the fact that some of those who were in favour of them were too ambitious as to when these technologies should be applied, and gave too little emphasis to other competing approaches which might have been more useful.

Environmental biotechnology has now fully regained its reputation, due to the hard work of skilled and dedicated scientists. Reliable documentation within a number of areas is rapidly accumulating, and new emerging approaches and tools are distinguishing the field. For these reasons, this book is extremely well timed.

The book covers both the basic fundamentals and biochemical processes involved, as well as the technologies themselves within different areas of application. As part of the framework, it also provides a thorough description of the character of pollution and pollution control, and there are chapters on more modern approaches to the subject, such as integrated environmental biotechnology and genetic tools – all in all a complete introduction to the study of environmental biotechnology.

There is no doubt that this book will be one of inspiration for all professionals in the field. It is a very good framework for understanding the complex nature of processes and technology, and as such it will be useful for researchers, practitioners and other parties who need a working knowledge of this fascinating subject.

Preface

This work inevitably sprang out of our environmental biotechnology modules at the University of Durham, but it is not intended to be just another 'book of the course'. Though it is clearly rooted in these origins, it reflects our wider, and rather varied, experiences of the field. In many respects, we have been fortunate; teaching has undoubtedly drawn on the 'theory', while our own consultancy has tended to focus us on the 'application'. Indeed, our own particular backgrounds mean that our partnership is based in both the academic and the practical. Like many before us, we came to the subject largely by accident and via other original disciplines, in the days before educational institutions offered anything other than traditional programmes of study and, please remember, this was not so long ago. The rise of environmental studies, which must surely be amongst the most inherently applicable of applied sciences, and the growing importance of biotechnology usage in this respect, remain two of the most encouraging developments for the future of our planet.

Within a very short time, biotechnology has come to play an increasingly important role in many aspects of everyday life. The upsurge of the 'polluter pays' principle, increasing pressure to revitalise the likes of former industrial sites and recent developments within the waste industry itself have combined to alter the viability of environmental biotechnology radically in the last five years. Once an expensive and largely unfamiliar option, it has now become a realistic alternative to many established approaches for manufacturing, land remediation, pollution control and waste management. Against a background of burgeoning disposal costs and ever more stringent legislation and liabilities, the application of biologically engineered solutions seems certain to continue its growth.

The purpose of this book is a straightforward one: to present a fair reflection of the practical biological approaches currently employed to address environmental problems, and to provide the reader with a working knowledge of the science that underpins them. In this respect, it differs very little from the ethos of our course at Durham and we are grateful to each successive wave of students for constantly reminding us of the importance of these two goals. In other ways, this work represents a major departure. Freed from the constraints of time and the inevitable demands of exams, we have been afforded the luxury in this book of being able to include far more in each section than could reasonably be covered in a traditional series of lectures on the topic. In some places, this has allowed

us to delve in deeper detail, while in others it has permitted some of the lesser well-known aspects of this fascinating discipline to be aired anew.

We have adopted what we feel is a logical structure, addressing technologies in as cohesive a manner as possible, given the intrinsic interrelatedness of so much of our subject matter. While the fundamental structure is, of course, intended to unify the whole work, we have tried to make each chapter as much of a 'standalone' as possible, in an attempt to make this a book which also encourages 'dipping in'. Ultimately, of course, the reader will decide how successful we have been.

The text falls into three main parts. The early chapters examine issues of the role and market for biotechnology in an environmental context, the essential biochemistry and microbiology which enables them to be met, and the fundamental themes of biological intervention. The technologies and applications themselves make up the central core of the book, both literally and figuratively and, fittingly, this is the largest part. Finally, aspects of integration and the future development of environmental biotechnology are addressed.

This subject is inherently context-dependent – a point which recurs throughout the discussion – and local modalities can conspire to shape individual best practice in a way unknown in other branches of biotechnology. What works in one country may not in another, not because the technology is flawed, but often simply because economic, legislative or societal barriers so dictate. The environmental biotechnologist must sometimes perform the mental equivalent of a circus act in balancing these many and different considerations. It is only to be expected, then, that the choices we have made as to what to include, and the relative importance afforded them, reflect these experiences. It is equally inevitable that some readers will take issue with these decisions, but that has always been the lot of writers. As an editor of our acquaintance once confided, the most powerful drive known to our species is not for survival, nor to procreate, but to alter someone else's copy.

It has been said that the greatest thing that anyone can achieve is to make a difference. We hope that, in writing this book, we will, in some small way, do just that.

Acknowledgements

The authors of any book always owe a debt of thanks to many people. Not in the slightly sycophantic way of the film awards, but in a very real sense, there truly are those without whom it would not have been possible to get the job done. The writers of this book are no exception and would like to say a public thank you to everyone who helped us along the way. To single anyone out always runs the risk of being divisive, but to omit a few particular individuals would be churlish in the extreme. We are particularly grateful to Lynne and David Lewis-Saunders for the use of our compact and bijou residence in the Dales, where so much of this book was written and to Linda Ormiston, OBE, for the loan of her coffee table, where most of the rest of it took shape.

We are, of course, terribly aware of the loss of the late Professor Peter Evans and enormously grateful to him for encouraging us to build up the environmental biotechnology course. He was very supportive of the wider objectives of this present work and it is a cause of much sadness that he will not see its publication. Our thoughts are with Di: both she and the University of Durham lost a thoroughly good man.

Thanks must also go to old friends – John Eccles, Rob Heap and Bob Talbott – for their assistance and to David Swan, Bob Rust, Graham Tebbitt, Vanessa Trescott and Bob Knight, for helping to get various facts and figures straight and in time for our deadline. Keily Larkins and Lyn Roberts of John Wiley & Sons Ltd have played a great game throughout. Always helpful and supportive, between them they have made contact often enough to reassure themselves that things really were progressing, but not so often as to intrude. This must be an awfully difficult balancing act and they have managed it very well.

We also know, to use the oft-quoted statement of Newton, that we stand on the shoulders of giants; that whatever knowledge we may possess and hopefully impart in this book, was gained thanks to those who have travelled this route before us. The debt to the great biologists, biochemists and engineers is clear, but it exists just as much to our own teachers who inspired us, to our contemporaries who spurred us on and to our parents without whom, quite literally, none of this would have been possible.

To all of these people we are deeply grateful for their help and support, as well as to our dogs, Mungo and Megan, for being quite so forgiving when the need to finish another chapter meant that their walks had to be curtailed.

1
Introduction to Biotechnology

The *Chambers Science and Technology Dictionary* defines biotechnology as 'the use of organisms or their components in industrial or commercial processes, which can be aided by the techniques of genetic manipulation in developing e.g. novel plants for agriculture or industry.' Despite the inclusiveness of this definition, the biotechnology sector is still often seen as largely medical or pharmaceutical in nature, particularly amongst the general public. While to some extent the huge research budgets of the drug companies and the widespread familiarity of their products makes this understandable, it does distort the full picture and somewhat unfairly so. However, while therapeutic instruments form, in many respects, the 'acceptable' face of biotechnology, elsewhere the science is all too frequently linked with unnatural interference. While the agricultural, industrial and environmental applications of biotechnology are potentially very great, the shadow of Frankenstein has often been cast across them. Genetic engineering may be relatively commonplace in pharmaceutical thinking and yet in other spheres, like agriculture for example, society can so readily and thoroughly demonise it.

The history of human achievement has always been episodic. For a while, one particular field of endeavour seems to hold sway as the preserve of genius and development, before the focus shifts and development forges ahead in dizzy exponential rush in an entirely new direction. So it was with art in the renaissance, music in the 18th century, engineering in the 19th and physics in the 20th. Now it is the age of the biological, possibly best viewed almost as a rebirth, after the great heyday of the Victorian naturalists, who provided so much input into the developing science. It is then, perhaps, no surprise that the European Federation of Biotechnology begins its 'Brief History' of the science in the year 1859, with the publication of *On the Origin of Species by Means of Natural Selection* by Charles Darwin. Though his famous voyage aboard *HMS Beagle*, which led directly to the formulation of his (then) revolutionary ideas, took place when he was a young man, he had delayed making them known until 1858, when he made a joint presentation before the Linnaean Society with Alfred Russell Wallace, who had, himself, independently come to very similar conclusions. Their contribution was to view evolution as the driving force of life, with successive selective pressures over time endowing living beings with optimised characteristics for survival. Neo-Darwinian thought sees the interplay of mutation and

natural selection as fundamental. The irony is that Darwin himself rejected mutation as too deleterious to be of value, seeing such organisms, in the language of the times, as 'sports' – oddities of no species benefit. Indeed, there is considerable evidence to suggest that he seems to have espoused a more Lamarckist view of biological progression, in which physical changes in an organism's lifetime were thought to shape future generations. Darwin died in 1882. Ninety-nine years after his death, the first patent for a genetically modified organism was granted to Ananda Chakrabarty of the US General Electric, relating to a strain of *Pseudomonas aeruginosa* engineered to express the genes for certain enzymes in order to metabolise crude oil. Twenty years later still, in the year that saw the first working draft of the human genome sequence published and the announcement of the full genetic blueprint of the fruit fly, *Drosophila melanogaster*, that archetype of eukaryotic genetics research, biotechnology has become a major growth industry with increasing numbers of companies listed on the world's stock exchanges. Thus, at the other end of the biotech timeline, a century and a half on from *Origin of Species*, the principles it first set out remain of direct relevance for what has been termed the 'chemical evolution' of biologically active substances and are commonly used in laboratories for *in vitro* production of desired qualities in biomolecules.

The Role of Environmental Biotechnology

While pharmaceutical biotechnology represents the glamorous end of the market, environmental applications are decidedly more in the Cinderella mould. The reasons for this are fairly obvious. The prospect of a cure for the many diseases and conditions currently promised by gene therapy and other biotech-oriented medical miracles can potentially touch us all. Our lives may, quite literally, be changed. Environmental biotechnology, by contrast, deals with far less apparently dramatic topics and, though their importance, albeit different, may be every bit as great, their direct relevance is far less readily appreciated by the bulk of the population. Cleaning up contamination and dealing rationally with wastes is, of course, in everybody's best interests, but for most people, this is simply addressing a problem which they would rather had not existed in the first place. Even for industry, though the benefits may be noticeable on the balance sheet, the likes of effluent treatment or pollution control are more of an inevitable obligation than a primary goal in themselves. In general, such activities are typically funded on a distinctly limited budget and have traditionally been viewed as a necessary inconvenience. This is in no way intended to be disparaging to industry; it simply represents commercial reality.

In many respects, there is a logical fit between this thinking and the aims of environmental biotechnology. For all the media circus surrounding the grand questions of our age, it is easy to forget that not all forms of biotechnology involve xenotransplantation, genetic modification, the use of stem cells or cloning.

Some of the potentially most beneficial uses of biological engineering, and which may touch the lives of the majority of people, however indirectly, involve much simpler approaches. Less radical and showy, certainly, but powerful tools, just the same. Environmental biotechnology is fundamentally rooted in waste, in its various guises, typically being concerned with the remediation of contamination caused by previous use, the impact reduction of current activity or the control of pollution. Thus, the principal aims of this field are the manufacture of products in environmentally harmonious ways, which allow for the minimisation of harmful solids, liquids or gaseous outputs or the clean-up of the residual effects of earlier human occupation.

The means by which this may be achieved are essentially two-fold. Environmental biotechnologists may enhance or optimise conditions for existing biological systems to make their activities happen faster or more efficiently, or they resort to some form of alteration to bring about the desired outcome. The variety of organisms which may play a part in environmental applications of biotechnology is huge, ranging from microbes through to trees and all are utilised on one of the same three fundamental bases – accept, acclimatise or alter. For the vast majority of cases, it is the former approach, accepting and making use of existing species in their natural, unmodified form, which predominates.

The Scope for Use

There are three key points for environmental biotechnology interventions, namely in the manufacturing process, waste management or pollution control, as shown in Figure 1.1.

Accordingly, the range of businesses to which environmental biotechnology has potential relevance is almost limitless. One area where this is most apparent is with regard to waste. All commercial operations generate waste of one form or another and for many, a proportion of what is produced is biodegradable. With disposal costs rising steadily across the world, dealing with refuse constitutes an increasingly high contribution to overheads. Thus, there is a clear incentive for all businesses to identify potentially cost-cutting approaches to waste and

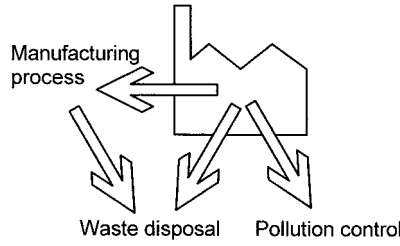

Figure 1.1 *The three intervention points*

employ them where possible. Changes in legislation throughout Europe, the US and elsewhere, have combined to drive these issues higher up the political agenda and biological methods of waste treatment have gained far greater acceptance as a result. For those industries with particularly high biowaste production, the various available treatment biotechnologies can offer considerable savings.

Manufacturing industries can benefit from the applications of whole organisms or isolated biocomponents. Compared with conventional chemical processes, microbes and enzymes typically function at lower temperatures and pressures. The lower energy demands this makes leads to reduced costs, but also has clear benefits in terms of both the environment and workplace safety. Additionally, biotechnology can be of further commercial significance by converting low-cost organic feedstocks into high value products or, since enzymatic reactions are more highly specific than their chemical counterparts, by deriving final substances of high relative purity. Almost inevitably, manufacturing companies produce wastewaters or effluents, many of which contain biodegradable contaminants, in varying degrees. Though traditional permitted discharges to sewer or watercourses may be adequate for some, other industries, particularly those with recalcitrant or highly concentrated effluents, have found significant benefits to be gained from using biological treatment methods themselves on site. Though careful monitoring and process control are essential, biotechnology stands as a particularly cost-effective means of reducing the pollution potential of wastewater, leading to enhanced public relations, compliance with environmental legislation and quantifiable cost-savings to the business.

Those involved in processing organic matter, for example, or with drying, printing, painting or coating processes, may give rise to the release of volatile organic compounds (VOCs) or odours, both of which represent environmental nuisances, though the former is more damaging than the latter. For many, it is not possible to avoid producing these emissions altogether, which leaves treating them to remove the offending contaminants the only practical solution. Especially for relatively low concentrations of readily water-soluble VOCs or odorous chemicals, biological technologies can offer an economic and effective alternative to conventional methods.

The use of biological cleaning agents is another area of potential benefit, especially where there is a need to remove oils and fats from process equipment, work surfaces or drains. Aside from typically reducing energy costs, this may also obviate the need for toxic or dangerous chemical agents. The pharmaceutical and brewing industries, for example, both have a long history of employing enzyme-based cleaners to remove organic residues from their process equipment. In addition, the development of effective biosensors, powerful tools which rely on biochemical reactions to detect specific substances, has brought benefits to a wide range of sectors, including the manufacturing, engineering, chemical, water, food and beverage industries. With their ability to detect even small amounts of their particular target chemicals, quickly, easily and accurately, they have

been enthusiastically adopted for a variety of process monitoring applications, particularly in respect of pollution assessment and control.

Contaminated land is a growing concern for the construction industry, as it seeks to balance the need for more houses and offices with wider social and environmental goals. The reuse of former industrial sites, many of which occupy prime locations, may typically have associated planning conditions attached which demand that the land be cleaned up as part of the development process. With urban regeneration and the reclamation of 'brown-field' sites increasingly favoured in many countries over the use of virgin land, remediation has come to play a significant role and the industry has an ongoing interest in identifying cost-effective methods of achieving it. Historically, much of this has involved simply digging up the contaminated soil and removing it to landfill elsewhere. Bioremediation technologies provide a competitive and sustainable alternative and in many cases, the lower disturbance allows the overall scheme to make faster progress.

As the previous brief examples show, the range of those which may benefit from the application of biotechnology is lengthy and includes the chemical, pharmaceutical, water, waste management and leisure industries, as well as manufacturing, the military, energy generation, agriculture and horticulture. Clearly, then, this may have relevance to the viability of these ventures and, as was mentioned at the outset, biotechnology is an essentially commercial activity. Environmental biotechnology must compete in a world governed by the *best practicable environmental option* (BPEO) and the *best available techniques not entailing excessive cost* (BATNEEC). Consequently, the economic aspect will always have a large influence on the uptake of all initiatives in environmental biotechnology and, most particularly, in the selection of methods to be used in any given situation. It is impossible to divorce this context from the decision-making process. By the same token, the sector itself has its own implications for the wider economy.

The Market for Environmental Biotechnology

The UK's Department of Trade and Industry estimated that 15–20% of the global environmental market in 2001 was biotech-based, which amounted to about 250–300 billion US dollars and the industry is projected to grow by as much as ten-fold over the following five years. This expected growth is due to greater acceptance of biotechnology for clean manufacturing applications and energy production, together with increased landfill charges and legislative changes in waste management which also alter the UK financial base favourably with respect to bioremediation. Biotechnology-based methods are seen as essential to help meet European Union (EU) targets for biowaste diversion from landfill and reductions in pollutants. Across the world the existing regulations on environmental pollution are predicted to be more rigorously enforced, with more

stringent compliance standards implemented. All of this is expected to stimulate the sales of biotechnology-based environmental processing methods significantly and, in particular, the global market share is projected to grow faster than the general biotech sector trend, in part due to the anticipated large-scale EU aid for environmental clean-up in the new accession countries of Eastern Europe.

Other sources paint a broadly similar picture. The BioIndustry Association (BIA) survey, *Industrial Markets for UK Biotechnology – Trends and Issues*, published in 1999 does not quote any monetary sector values per year, but gives the size of the UK sector as employing 40 000 people in 1998 with an average yearly growth over 1995–98 of 20%. Environmental biotech is reported as representing around 10% of this sector. An Arthur Anderson report of 1997 gives the turnover of the UK biotech sector as 702 million pounds sterling in 1995/96, with a 50% growth over three years. A 1998 Ernst and Young report on the European Life Sciences Sector says that the market for biotechnology products has the potential to reach 100 billion pounds sterling worldwide by 2005. The Organisation for Economic Cooperation and Development (OECD) estimates that the global market for environmental biotechnology products and services alone will rise to some US$75 billion by the year 2000, accounting for some 15 to 25% of the overall environmental technology market, which has a growth rate estimated at 5.5% per annum. The UK potential market for environmental biotechnology products and services is estimated at between 1.65 and 2.75 billion US dollars and the growth of the sector stands at 25% per annum, according to the *Bio-Commerce Data European Biotechnology Handbook*. An unsourced quote found on a Korean University website says that the world market size of biotechnology products and services was estimated to be approximately 390 billion US dollars in the year 2000.

The benefits are not, however, confined to the balance sheet. The Organisation for Economic Cooperation and Development (OECD 2001) concluded that the industrial use of biotechnology commonly leads to increasingly environmentally harmonious processes and additionally results in lowered operating and/or capital costs. For years, industry has appeared locked into a seemingly unbreakable cycle of growth achieved at the cost of environmental damage. The OECD investigation provides what is probably the first hard evidence to support the reality of biotechnology's long-heralded promise of alternative production methods, which are ecologically sound and economically efficient. A variety of industrial sectors including pharmaceuticals, chemicals, textiles, food and energy were examined, with a particular emphasis on biomass renewable resources, enzymes and bio-catalysis. While such approaches may have to be used in tandem with other processes for maximum effectiveness, it seems that their use invariably leads to reduction in operating or capital costs, or both. Moreover, the research also concludes that it is clearly in the interests of governments of the developed and developing worlds alike to promote the use of biotechnology for the substantial reductions in resource and energy consumption, emissions, pollution and waste production

it offers. The potential contribution to be made by the appropriate use of biotechnology to environmental and economic sustainability would seem to be clear.

The upshot of this is that few biotech companies in the environmental sector perceive problems for their own business development models, principally as a result of the wide range of businesses for which their services are applicable, the relatively low market penetration to date and the large potential for growth. Competition within the sector is not seen as a major issue either, since the field is still largely open and unsaturated. Moreover, there has been a discernible tendency in recent years towards niche specificity, with companies operating in more specialised subarenas within the environmental biotechnology umbrella. Given the number and diversity of such possible slots, coupled with the fact that new opportunities, and the technologies to capitalise on them, are developing apace, this trend seems likely to continue. It is not without some irony that companies basing their commercial activities on biological organisms should themselves come to behave in such a Darwinian fashion. However, the picture is not entirely rosy.

Typically the sector comprises a number of relatively small, specialist companies and the market is, as a consequence, inevitably fragmented. Often the complexities of individual projects make the application of 'standard' off-the-shelf approaches very difficult, the upshot being that much of what is done must be significantly customised. While this, of course, is a strength and of great potential environmental benefit, it also has hard commercial implications which must be taken into account. A sizeable proportion of companies active in this sphere, have no products or services which might reasonably be termed suitable for generalised use, though they may have enough expertise, experience or sufficiently perfected techniques to deal with a large number of possible scenarios. The fact remains that one of the major barriers to the wider uptake of biological approaches is the high perceived cost of these applications. Part of the reason for this lies in historical experience. For many years, the solutions to all environmental problems were seen as expensive and for many, particularly those unfamiliar with the multiplicity of varied technologies available, this has remained the prevalent view. Generally, there is often a lack of financial resource allocation available for this kind of work and biotech providers have sometimes come under pressure to reduce the prices for their services as a result. Greater awareness of the benefits of biotechnology, both as a means to boost existing markets and for the opening up of new ones, is an important area to be addressed. Many providers, particularly in the UK, have cited a lack of marketing expertise as one of the principal barriers to their exploitation of novel opportunities. In addition, a lack of technical understanding of biotech approaches amongst target industries and, in some cases, downright scepticism regarding their efficacy, can also prove problematic. Good education, in the widest sense, of customers and potential users of biological solutions will be one major factor in any future upswing in the acceptance and utilisation of these technologies.

Modalities and local influences

Another of the key factors affecting the practical uptake of environmental biotechnology is the effect of local circumstances. Contextual sensitivity is almost certainly the single most important factor in technology selection and represents a major influence on the likely penetration of biotech processes into the marketplace. Neither the nature of the biological system, nor of the application method itself, play anything like so relevant a role. This may seem somewhat unexpected at first sight, but the reasons for it are obvious on further inspection. While the character of both the specific organisms and the engineering remain essentially the same irrespective of location, external modalities of economics, legislation and custom vary on exactly this basis. Accordingly, what may make abundant sense as a biotech intervention in one region or country, may be totally unsuited to use in another. In as much as it is impossible to discount the wider global economic aspects in the discussion, disassociating political, fiscal and social conditions equally cannot be done, as the following example illustrates. In 1994, the expense of bioremediating contaminated soil in the United Kingdom greatly exceeded the cost of removing it to landfill. Six years later, with successive changes of legislation and the imposition of a landfill tax, the situation has almost completely reversed. In those other countries where landfill has always been an expensive option, remediation has been embraced far more readily.

While environmental biotechnology must, inevitably, be viewed as contextually dependent, as the previous example shows, contexts can change. In the final analysis, it is often fiscal instruments, rather than the technologies, which provide the driving force and sometimes seemingly minor modifications in apparently unrelated sectors can have major ramifications for the application of biotechnology. Again as has been discussed, the legal framework is another aspect of undeniable importance in this respect. Increasingly tough environmental law makes a significant contribution to the sector and changes in regulatory legislation are often enormously influential in boosting existing markets or creating new ones. When legislation and economic pressure combine, as, for example, they have begun to do in the European Landfill Directive, the impetus towards a fundamental paradigm shift becomes overwhelming and the implications for relevant biological applications can be immense.

There is a natural tendency to delineate, seeking to characterise technologies into particular categories or divisions. However, the essence of environmental biotechnology is such that there are many more similarities than differences. Though it is, of course, often helpful to view individual technology uses as distinct, particularly when considering treatment options for a given environmental problem, there are inevitably recurrent themes which feature throughout the whole topic. Moreover, this is a truly applied science. While the importance of the laboratory bench cannot be denied, the controlled world of research translates imperfectly into the harsh realities of commercial implementation. Thus, there can often be a dichotomy between theory and application and it is precisely

this fertile ground which is explored in the present work. In addition, the principal underlying approach of specifically *environmental* biotechnology, as distinct from other kinds, is the reliance on existing natural cycles, often directly and in an entirely unmodified form. Thus, this science stands on a foundation of fundamental biology and biochemistry. To understand the application, the biotechnologist must simply examine the essential elements of life, living systems and ecological circulation sequences. However engineered the approach, this fact remains true. In many respects, environmental biotechnology stands as the purest example of the newly emergent bioindustry, since it is the least refined, at least in terms of the basis of its action. In essence, all of its applications simply encourage the natural propensity of the organisms involved, while seeking to enhance or accelerate their action. Hence, optimisation, rather than modification, is the typical route by which the particular desired end result, whatever it may be, is achieved and, consequently, a number of issues feature as common threads within the discussions of individual technologies.

Integrated Approach

Integration is an important aspect for environmental biotechnology. One theme that will be developed throughout this book is the potential for different biological approaches to be combined within treatment trains, thereby producing an overall effect which would be impossible for any single technology alone to achieve. However, the wider goal of integration is not, of necessity, confined solely to the specific methods used. It applies equally to the underpinning knowledge that enables them to function in the first place and an understanding of this is central to the rationale behind this book. In some spheres, traditional biology has become rather unfashionable and the emphasis has shifted to more exciting sounding aspects of life science. While the new-found concentration on 'ecological processes', or whatever, sounds distinctly more 'environmental', in many ways, and somewhat paradoxically, it sometimes serves the needs of environmental biotechnology rather less well. The fundamentals of living systems are the stuff of this branch of science and, complex though the whole picture may be, at its simplest the environmental biotechnologist is principally concerned with a relatively small number of basic cycles. In this respect, a good working knowledge of biological processes like respiration, fermentation and photosynthesis, a grasp of the major cycles by which carbon, nitrogen and water are recycled and an appreciation of the flow of energy through the biosphere must be viewed as prerequisites. Unsurprisingly, then, these basic processes appear throughout this book, either explicitly or tacitly accepted as underpinning the context of the discussion. The intent here has been neither to insult the readership by parading what is already well known, nor gloss over aspects which, if left unexplained, at least in reasonable detail, might only serve to confuse. However, this is expressly not designed to be a substitute for much more specific texts on these subjects, nor

an entire alternative to a cohesive course on biology or biochemistry. The intention is to introduce and explain the necessary aspects and elements of various metabolic pathways, reactions and abilities as required to advance the reader's understanding of this particular branch of biotechnology.

A large part of the reasons for approaching the subject in this way is the fact that there really is no such thing as a 'typical' environmental biotechnologist. Practitioners come into the profession from a wide variety of disciplines and by many different routes. Thus, amongst their ranks are agronomists, biochemists, biologists, botanists, enzymologists, geneticists, microbiologists, molecular biologists, process engineers and protein technologists, all of whom bring their own particular skills, knowledge base and experiences. The applied nature of environmental biotechnology is obvious. While the science underlying the processes themselves may be as pure as any other, what distinguishes this branch of biological technology are the distinctly real-life purposes to which it is put. Hence, part of the intended function of this book is to attempt to elucidate the former in order to establish the basis of the latter. At the same time, as any applied scientist will confirm, what happens in the field under operational conditions represents a distinct compromise between the theoretical and the practically achievable. At times, anything more than an approximation to the expected results may be counted as something of a triumph of environmental engineering.

Closing Remarks

The celebrated astronomer and biologist, Sir Fred Hoyle, said that the solutions to major unresolved problems should be sought by the exploration of radical hypotheses, while simultaneously adhering to well-tried and tested scientific tools and methods. This approach is particularly valid for environmental biotechnology. With new developments in treatment technologies appearing all the time, the list of what can be processed or remediated by biological means is ever changing. By the same token, the applications for which biotechnological solutions are sought are also subject to alteration. For the biotech sector to keep abreast of these new demands it may be necessary to examine some truly 'radical hypotheses' and possibly make use of organisms or their derivatives in ways previously unimagined. This is the basis of innovation; the inventiveness of an industry is often a good measure of its adaptability and commercial robustness.

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2
Microbes and Metabolism

So fundamental are the concepts of cell growth and metabolic capability to the whole of environmental biotechnology and especially to remediation, that this chapter is dedicated to their exploration. Metabolic pathways (Michal 1992) are interlinked to produce what can develop into an extraordinarily complicated network, involving several levels of control. However, they are fundamentally about the interaction of natural cycles and represent the biological element of the natural geobiological cycles. These impinge on all aspects of the environment, both living and nonliving. Using the carbon cycle as an example, carbon dioxide in the atmosphere is returned by dissolution in rainwater, and also by the process of photosynthesis to produce sugars, which are eventually metabolised to liberate the carbon once more. In addition to constant recycling through metabolic pathways, carbon is also sequestered in living and nonliving components such as in trees in the relatively short term, and deep ocean systems or ancient deposits, such as carbonaceous rocks, in the long term. Cycles which involve similar principles of incorporation into biological molecules and subsequent re-release into the environment operate for nitrogen, phosphorus and sulphur. All of these overlap in some way, to produce the metabolic pathways responsible for the synthesis and degradation of biomolecules. Superimposed, is an energy cycle, ultimately driven by the sun, and involving constant consumption and release of metabolic energy.

To appreciate the biochemical basis and underlying genetics of environmental biotechnology, at least an elementary grasp of molecular biology is required. For the benefit of readers unfamiliar with these disciplines, background information is incorporated in appropriate figures.

The Immobilisation, Degradation or Monitoring of Pollutants from a Biological Origin

Removal of a material from an environment takes one of two routes: it is either degraded or immobilised by a process which renders it biologically unavailable for degradation and so is effectively removed.

Immobilisation can be achieved by chemicals excreted by an organism or by chemicals in the neighbouring environment which trap or chelate a molecule thus making it insoluble. Since virtually all biological processes require the substrate to be dissolved in water, chelation renders the substance unavailable. In some instances

this is a desirable end result and may be viewed as a form of remediation, since it stabilises the contaminant. In other cases it is a nuisance, as digestion would be the preferable option. Such 'unwanted' immobilisation can be a major problem in remediation, and is a common state of affairs with aged contamination. Much research effort is being applied to find methods to reverse the process.

Degradation is achieved by metabolic pathways operating within an organism or combination of organisms, sometimes described as consortia. These processes are the crux of environmental biotechnology and thus form the major part of this chapter. Such activity operates through metabolic pathways functioning within the cell, or by enzymes either excreted by the cell or, isolated and applied in a purified form.

Biological monitoring utilises proteins, of which enzymes are a subset, produced by cells, usually to identify, or quantify contaminants. This has recently developed into an expanding field of biosensor production.

Who are the biological players in these processes, what are their attributes which are so essential to this science and which types of biological material are being addressed here? The answers to these questions lie throughout this book and are summarised in this chapter.

The players

Traditionally, life was placed into two categories – those having a true *nucleus* (eukaryotes) and those that do not (prokaryotes). This view was dramatically disturbed in 1977 when Carl Woese proposed a third domain, the archaebacteria, now described as archaea, arguing that although apparently prokaryote at first glance they contain sufficient similarities with eukaryotes, in addition to unique features of their own, to merit their own classification (Woese and Fox 1977, Woese, Kandler and Wheelis 1990). The arguments raised by this proposal continue (Cavalier-Smith 2002) but throughout this book the classification adopted is that of Woese, namely, that there are three divisions: bacteria, archaea (which together comprise prokaryotes) and eukaryotes. By this definition, then, what are referred to throughout this work simply as 'bacteria' are synonymous with the term eubacteria (meaning 'true' bacteria).

It is primarily to the archaea, which typically inhabit extreme niches with respect to temperature, pressure, salt concentration or osmotic pressure, that a great debt of gratitude is owed for providing this planet with the metabolic capability to carry out processes under some very odd conditions indeed. The importance to environmental biotechnology of life in extreme environments is addressed in Chapter 3.

An appreciation of the existence of these classifications is important, as they differ from each other in the detail of their cell organization and cellular processes making it unlikely that their genes are directly interchangeable. The relevance of this becomes obvious when genetic engineering is discussed later in this book in Chapter 9. However, it is interesting to examine the potentially prokaryotic

origins of the eukaryotic cell. There are many theories but the one which appears to have the most adherents is the endosymbiotic theory. It suggests that the 'proto' eukaryotic cell lost its cell wall, leaving only a membrane, and phagocytosed or subsumed various other bacteria with which it developed a symbiotic relationship. These included an aerobic bacterium, which became a mitochondrion, endowing the cell with the ability to carry out oxidative phosphorylation, a method of producing chemical energy able to be transferred to the location in the cell where it is required. Similarly, the chloroplast, the site of photosynthesis in higher plants, is thought to have been derived from cyanobacteria, the so-called blue-green algae. Chloroplasts are a type of plastid. These are membrane-bound structures found in vascular plants. Far from being isolated cellular organelles, the plastids communicate with each other through interconnecting tubules (Köhler *et al.* 1997). Various other cellular appendages are also thought to have prokaryotic origins such as cilia or the flagellum on a motile eukaryotic cell which may have formed from the fusion of a spirochete bacterium to this 'proto' eukaryote. Nuclei may well have similar origins but the evidence is still awaited.

No form of life should be overlooked as having a potential part to play in environmental biotechnology. However, the organisms most commonly discussed in this context are microbes and certain plants. They are implicated either because they are present by virtue of being in their natural environment or by deliberate introduction.

Microbes

Microbes are referred to as such, simply because they cannot be seen by the naked eye. Many are bacteria or archaea, all of which are prokaryotes, but the term 'microbe' also encompasses some eukaryotes, including yeasts, which are unicellular fungi, as well as protozoa and unicellular plants. In addition, there are some microscopic multicellular organisms, such as rotifers, which have an essential role to play in the microsystem ecology of places such as sewage treatment plants. An individual cell of a eukaryotic multicellular organism like a higher plant or animal, is approximately 20 microns in diameter, while a yeast cell, also eukaryotic but unicellular, is about five microns in diameter. Although bacterial cells occur in a variety of shapes and sizes, depending on the species, typically a bacterial cell is rod shaped, measuring approximately one micron in width and two microns in length. At its simplest visualisation, a cell, be it a unicellular organism, or one cell in a multicellular organism, is a bag, bounded by a membrane, containing an aqueous solution in which are all the molecules and structures required to enable its continued survival. In fact, this 'bag' represents a complicated infrastructure differing distinctly between prokaryotes and eukaryotes (Cavalier-Smith 2002), but a discussion of this is beyond the scope of this book.

Depending on the microbe, a variety of other structures may be present, for instance, a cell wall providing additional protection or support, or a flagellum,

a flexible tail, giving mobility through the surrounding environment. Survival requires cell growth, replication of the DNA and then division, usually sharing the contents into two equal daughter cells. Under ideal conditions of environment and food supply, division of some bacteria may occur every 20 minutes, however, most take rather longer. However, the result of many rounds of the binary division just described, is a colony of identical cells. This may be several millimetres across and can be seen clearly as a contamination on a solid surface, or if in a liquid, it will give the solution a cloudy appearance. Other forms of replication include budding off, as in some forms of yeast, or the formation of spores as in other forms of yeast and some bacteria. This is a type of DNA storage particularly resistant to environmental excesses of heat and pH, for example. When the environment becomes more hospitable, the spore can develop into a bacterium or yeast, according to its origins, and the life cycle continues.

Micro-organisms may live as free individuals or as communities, either as a clone of one organism, or as a mixed group. Biofilms are examples of microbial communities, the components of which may number several hundred species. This is a fairly loose term used to describe any aggregation of microbes which coats a surface, consequently, biofilms are ubiquitous. They are of particular interest in environmental biotechnology since they represent the structure of microbial activity in many relevant technologies such as trickling filters. Models for their organisation have been proposed (Kreft *et al*. 2001). Their structure, and interaction between their members, is of sufficient interest to warrant at least one major symposium (Allison *et al*. 2000). Commonly, biofilms occur at a solid/liquid interphase. Here, a mixed population of microbes live in close proximity which may be mutually beneficial. Such consortia can increase the habitat range, the overall tolerance to stress and metabolic diversity of individual members of the group. It is often thanks to such communities, rather than isolated bacterial species, that recalcitrant pollutants are eventually degraded due to combined contributions of several of its members.

Another consequence of this close proximity is the increased likelihood of bacterial transformation. This is a procedure whereby a bacterium may absorb free deoxyribonucleic acid (DNA), the macromolecule which stores genetic material, from its surroundings released by other organisms, as a result of cell death, for example. The process is dependent on the ability, or competence, of a cell to take up DNA, and upon the concentration of DNA in the surrounding environment. This is commonly referred to as horizontal transfer as opposed to vertical transfer which refers to inherited genetic material, either by sexual or asexual reproduction. Some bacteria are naturally competent, others exude competence factors and recently, there is laboratory evidence that lightning can impart competence to some bacteria (Demaneche *et al*. 2001). It is conceivable that conditions allowing transformation prevail in biofilms considering the very high local concentration of microbes. Indeed there is evidence that such horizontal transfer of DNA occurs between organisms in these communities (Ehlers 2000). In addition

to transformation, genes are readily transferred on plasmids as described later in this chapter. It is now well established that, by one method or another, there is so much exchange of genetic material between bacteria in soil or in aquatic environments, that rather than discrete units, they represent a massive gene pool (Whittam 1992).

The sliminess often associated with biofilms is usually attributed to excreted molecules often protein and carbohydrate in nature, which may coat and protect the film. Once established, the biofilm may proliferate at a rate to cause areas of anoxia at the furthest point from the source of oxygen, thus encouraging the growth of anaerobes. Consequently, the composition of the biofilm community is likely to change with time.

To complete the picture of microbial communities, it must be appreciated that they can include the other micro-organisms listed above, namely, yeasts, protozoa, unicellular plants and some microscopic multicellular organisms such as rotifers.

Plants

In contrast with microbes, the role of plants in environmental biotechnology is generally a structural one, exerting their effect by oxygenation of a microbe-rich environment, filtration, solid-to-gas conversion or extraction of the contaminant. These examples are examined in detail in Chapters 7 and 10. Genetic modification of crop plants to produce improved or novel varieties is discussed in Chapter 9. This field of research is vast and so the discussion is confined to relevant issues in environmental biotechnology rather than biotechnology in general.

Metabolism

The energy required to carry out all cellular processes is obtained from ingested food in the case of chemotrophic cells, additionally from light in the case of phototrophs and from inorganic chemicals in lithotrophic organisms. Since all biological macromolecules contain the element carbon, a dietary source of carbon is a requirement. Ingested food is therefore, at the very least, a source of energy and carbon, the chemical form of which is rearranged by passage through various routes called metabolic pathways. One purpose of this reshuffling is to produce, after addition or removal of other elements such as hydrogen, oxygen, nitrogen, phosphorous and sulphur, all the chemicals necessary for growth. The other is to produce chemical energy in the form of adenosine triphosphate (ATP), also one of the ‘building blocks’ of nucleic acids. Where an organism is unable to synthesise all its dietary requirements, it must ingest them, as they are, by definition, essential nutrients. The profile of these can be diagnostic for that organism and may be used in its identification in the laboratory. An understanding of nutritional requirements of any given microbe, can prove essential for successful remediation by bioenhancement.

At the core of metabolism are the central metabolic pathways of glycolysis and the tricarboxylic acid (TCA) cycle on which a vast array of metabolic pathways eventually converge or from which they diverge. Glycolysis is the conversion of the six-carbon phosphorylated sugar, glucose 6-phosphate, to the three-carbon organic acid, pyruvic acid, and can be viewed as pivotal in central metabolism since from this point, pyruvate may enter various pathways determined by the energy and synthetic needs of the cell at that time. A related pathway, sharing some but not all of the reactions of glycolysis, and which operates in the opposite direction is called gluconeogenesis. Pyruvate can continue into the TCA cycle whose main function is to produce and receive metabolic intermediates and to produce energy, or into one of the many fermentation routes.

The principles of glycolysis are universal to all organisms known to date, although the detail differs between species. An outline of glycolysis, the TCA, and its close relative the glyoxalate, cycles is given in Figure 2.1, together with an indication of the key points at which the products of macromolecule catabolism,

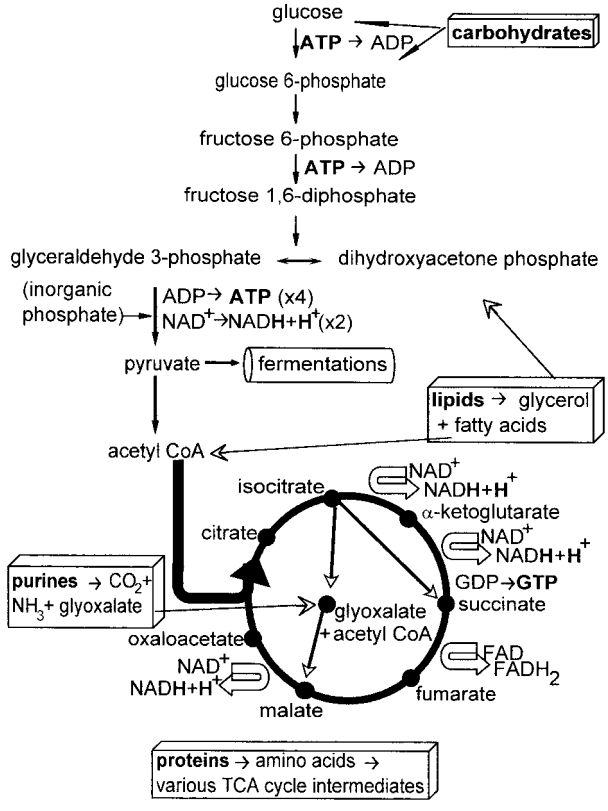


Figure 2.1 *Glycolysis, the TCA and glyoxalate cycles*

or breakdown, enter these central metabolic pathways. The focus is on degradation rather than metabolism in general, since this is the crux of bioremediation. A description of the biological macromolecules which are lipids, carbohydrates, nucleic acids and proteins are given in the appropriate figures (Figures 2.2–2.5).

Not all possible metabolic routes are present in the genome of any one organism. Those present are the result of evolution, principally of the enzymes which catalyse the various steps, and the elements which control their expression. However, an organism may have the DNA sequences, and so have the genetic capability for a metabolic route even though it is not 'switched on'. This is the basis for the description of 'latent pathways' which suggests the availability of a

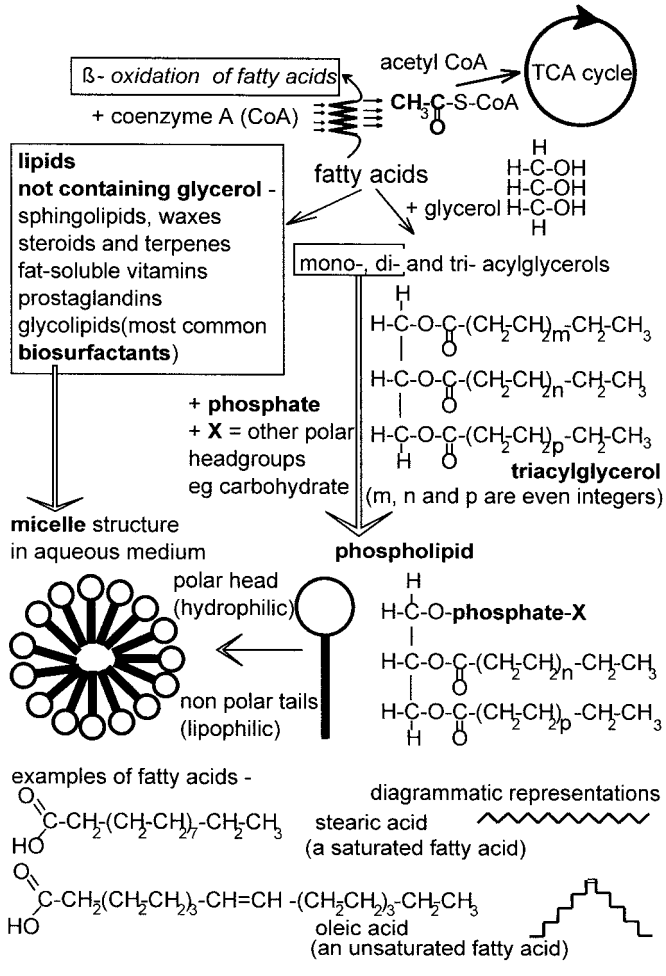


Figure 2.2 *Lipids*

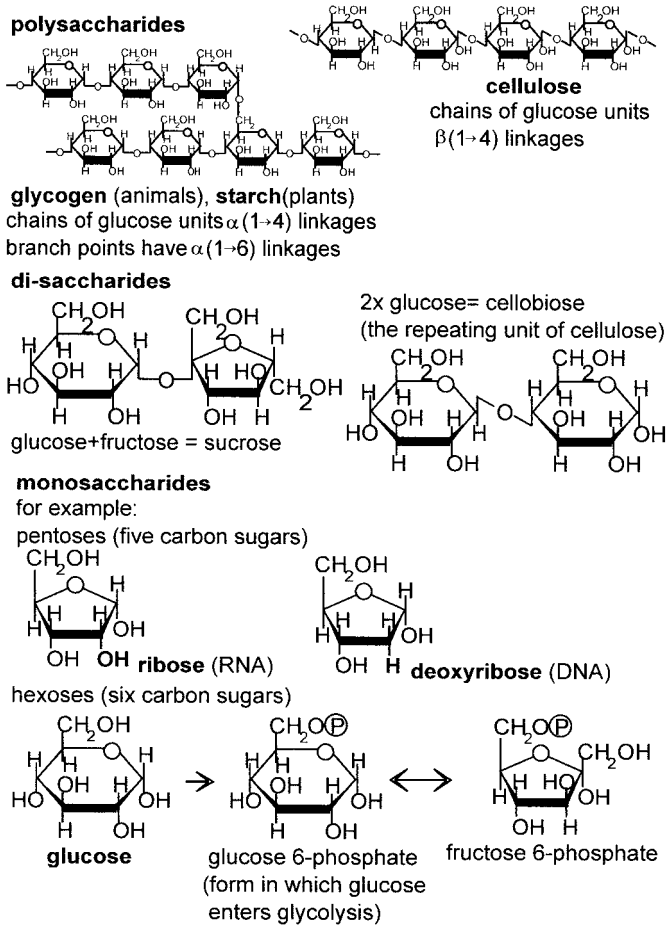


Figure 2.3 *Carbohydrates*

route able to be activated when the need arises, such as challenge from a novel chemical in the environment. Additionally, there is enormous potential for uptake and exchange of genetic information as discussed earlier in this chapter. It is the enormous range of metabolic capability which is harnessed in environmental biotechnology.

The basis of this discipline is about ensuring that suitable organisms are present which have the capability to perform the task required of them. This demands the provision of optimal conditions for growth, thus maximising degradation or removal of the contaminant. Linked to many of the catalytic steps in the metabolic pathway are reactions which release sufficient energy to allow the synthesis of ATP. This is the energy 'currency' of a cell which permits the transfer of energy

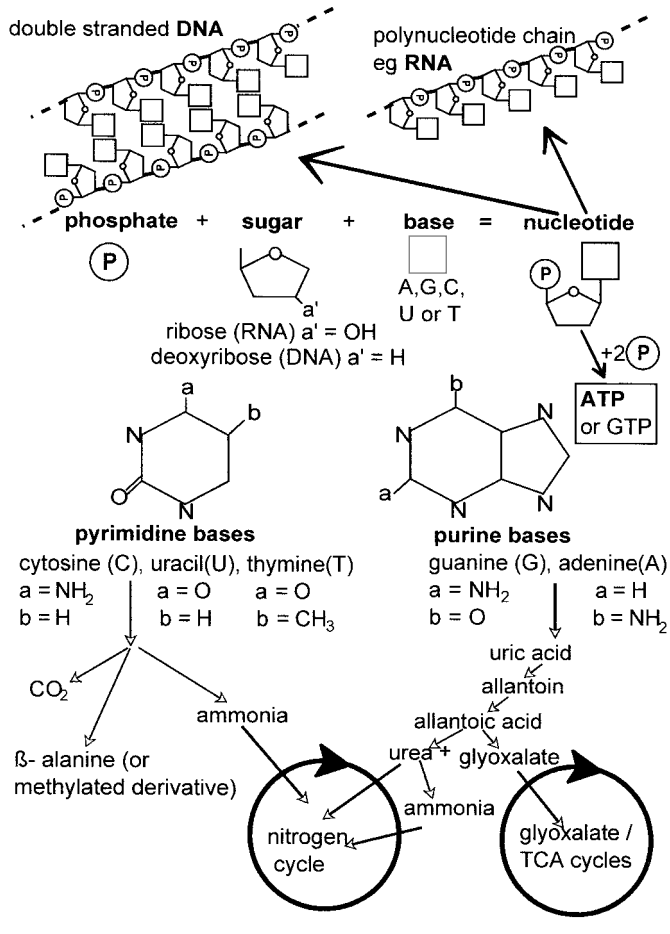


Figure 2.4 *Nucleic acids*

produced during degradation of a food to a process which may be occurring in a distant location and which requires energy.

For brevity, the discussions in this chapter consider the metabolic processes of prokaryotes and unicellular eukaryotes as equivalent to a single cell of a multicellular organism such as an animal or plant. This is a hideous oversimplification but justified when the points being made are general to all forms of life. Major differences are noted.

The genetic blueprint for metabolic capability

Metabolic capability is the ability of an organism or cell to digest available food. Obviously, the first requirement is that the food should be able to enter

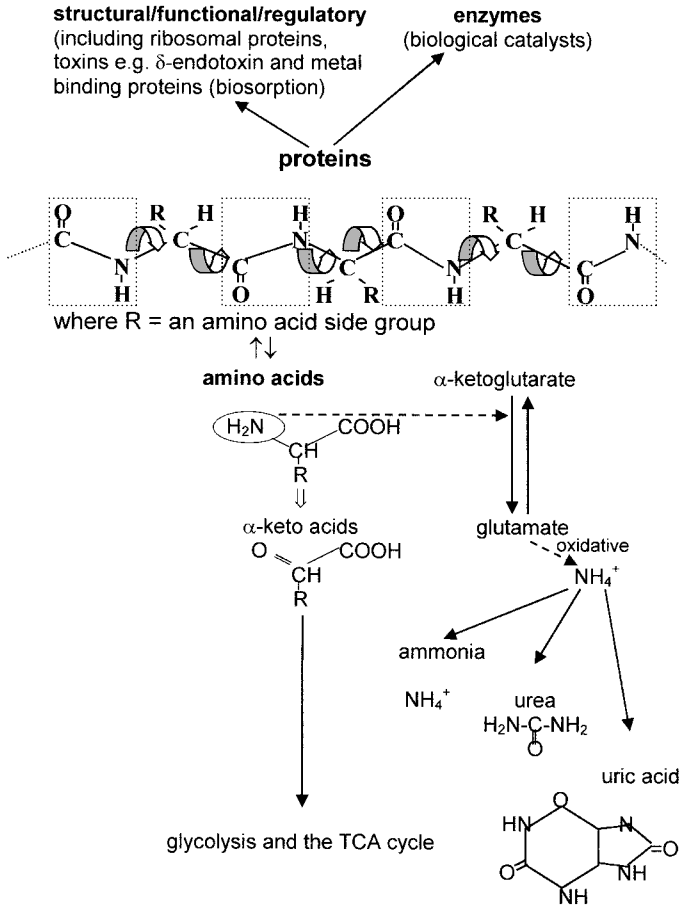


Figure 2.5 *Proteins*

the cell which sometimes requires specific carrier proteins to allow penetration across the cell membrane. Once entered, the enzymes must be present to catalyse all the reactions in the pathway responsible for degradation, or catabolism. The information for this metabolic capability, is encoded in the DNA. The full genetic information is described as the genome and can be a single circular piece of DNA as in bacteria, or may be linear and fragmented into chromosomes as in higher animals and plants.

Additionally, many bacteria carry plasmids, which are much smaller pieces of DNA, also circular and self-replicating. These are vitally important in the context of environmental biotechnology in that they frequently carry the genes for degradative pathways. Many of these plasmids may move between different

bacteria where they replicate, thus making the metabolic capability they carry, transferable. Bacteria show great promiscuity with respect to sharing their DNA. Often, bacteria living in a contaminated environment, themselves develop additional degradative capabilities. The source of that genetic information new to the organism, whether it is from modification of DNA within the organism or transfer from other microbes, or DNA free in the environment, is a source of hot debate between microbiologists.

DNA not only codes for RNA which is translated into proteins but also for RNAs which are involved in protein synthesis, namely transfer RNA (tRNA) and ribosomal RNA (rRNA), also, small RNAs which are involved in the processing of rRNA. These are illustrated in Figure 2.6. There have been many systems used to describe the degree of relatedness between organisms, but the most generally

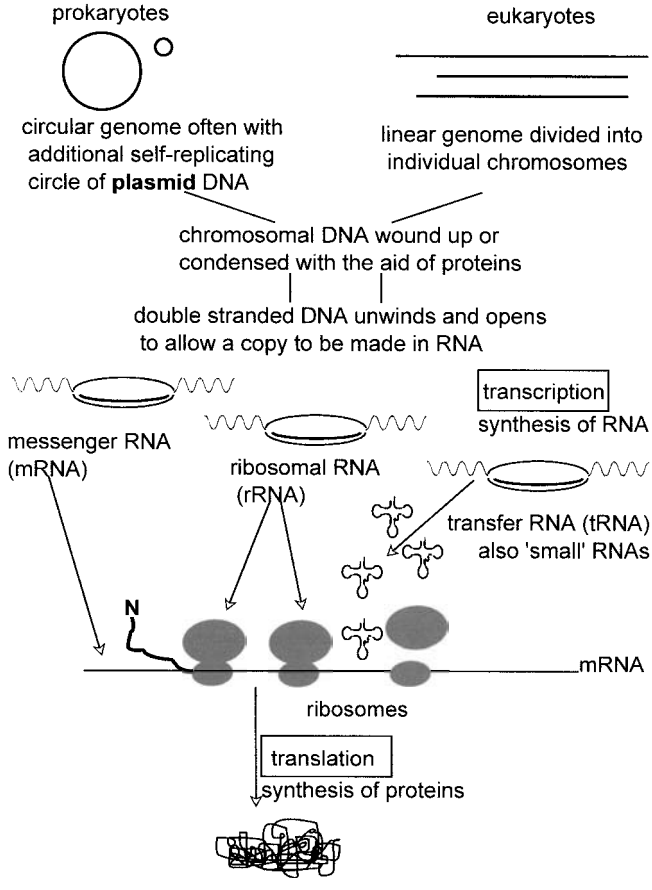

prokaryotes
eukaryotes
circular genome often with additional self-replicating circle of **plasmid** DNA
linear genome divided into individual chromosomes
chromosomal DNA wound up or condensed with the aid of proteins
double stranded DNA unwinds and opens to allow a copy to be made in RNA
transcription
synthesis of RNA
messenger RNA (mRNA)
ribosomal RNA (rRNA)
transfer RNA (tRNA) also 'small' RNAs
N
mRNA
ribosomes
translation
synthesis of proteins

Figure 2.6 *Storage and expression of genetic information*

accepted is based on the sequence of the DNA coding for ribosomal RNA, the rDNA (Stackebrandt and Woese 1981). For completeness, it is important to mention the retroviruses which are a group of eukaryotic viruses with RNA rather than DNA as their genome. They carry the potential for integration into inheritable DNA due to the way in which they replicate their genomic RNA by way of a DNA intermediate.

Microbial diversity

Microbes have been discovered in extraordinarily hostile environments where their continued survival has made demands on their structure and metabolic capability. These organisms, frequently members of the archaea, are those which have the capacity to degrade some of the most hazardous and recalcitrant chemicals in our environment and thus provide a rich source of metabolic capacity to deal with some very unpleasant contaminants. This situation will remain as long as the environments which harbour these invaluable microbes are recognised as such and are not destroyed. Microbial life on this planet, taken as a whole, has an immense capability to degrade noxious contaminants; it is essential to maintain the diversity and to maximise the opportunity for microbes to metabolise the offending carbon source.

Metabolic Pathways of Particular Relevance to Environmental Biotechnology

Having established that the overall strategy of environmental biotechnology is to make use of the metabolic pathways in micro-organisms to break down or metabolise organic material, this chapter now examines those pathways in some detail. Metabolic pathways operating in the overall direction of synthesis are termed anabolic while those operating in the direction of breakdown or degradation are described as catabolic: the terms catabolism and anabolism being applied to describe the degradative or synthetic processes respectively.

It has been mentioned already in this chapter and it will become clear from the forthcoming discussion, that the eventual fate of the carbon skeletons of biological macromolecules is entry into the central metabolic pathways.

Glycolysis

As the name implies, glycolysis is a process describing the splitting of a phosphate derivative of glucose, a sugar containing six carbon atoms, eventually to produce two pyruvate molecules, each having three carbon atoms. There are at least four pathways involved in the catabolism of glucose. These are the Embden–Meyerhof (Figure 2.1), which is the one most typically associated with glycolysis, the Entner–Doudoroff and the phosphoketolase pathways and the pentose phosphate

cycle, which allows rearrangement into sugars containing 3, 4, 5, 6 or 7 carbon atoms. The pathways differ from each other in some of the reactions in the first half up to the point of lysis to two three-carbon molecules, after which point the remainder of the pathways are identical. These routes are characterised by the particular enzymes present in the first half of these pathways catalysing the steps between glucose and the production of dihydroxyacetone phosphate in equilibrium with glyceraldehyde 3-phosphate. All these pathways have the capacity to produce ATP and so function in the production of cellular energy. The need for four different routes for glucose catabolism, therefore, lies in the necessity for the supply of different carbon skeletons for anabolic processes and also for the provision of points of entry to glycolysis for catabolites from the vast array of functioning catabolic pathways. Not all of these pathways operate in all organisms. Even when several are encoded in the DNA, exactly which of these are active in an organism at any time, depends on its current metabolic demands and the prevailing conditions in which the microbe is living.

The point of convergence of all four pathways is at the triose phosphates which is the point where glycerol as glycerol phosphate enters glycolysis and so marks the link between catabolism of simple lipids and the central metabolic pathways. The addition of glycerol to the pool of trioses is compensated for by the action of *triose phosphate isomerase* maintaining the equilibrium between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate which normally lies far in favour of the latter. This is perhaps surprising since it is glyceraldehyde 3-phosphate which is the precursor for the subsequent step. The next stage is the introduction of a second phosphate group to glyceraldehyde 3-phosphate with an accompanying oxidation, to produce glyceraldehyde 1,3-diphosphate. The oxidation involves the transfer of hydrogen to the coenzyme, NAD, to produce its reduced form, NADH. In order for glycolysis to continue operating, it is essential for the cell or organism to regenerate the NAD^+ which is achieved either by transfer of the hydrogens to the cytochromes of an electron transport chain whose operation is associated with the synthesis of ATP, or to an organic molecule such as pyruvate in which case the opportunity to synthesise ATP is lost. This latter method is the first step of many different fermentation routes. These occur when operation of electron transport chains is not possible and so become the only route for the essential regeneration of NAD^+. Looking at the Embden–Meyerhof pathway, this is also the third stage at which a phosphorylation has occurred. In this case, the phosphate was derived from an inorganic source, in a reaction which conserves the energy of oxidation.

The next step in glycolysis is to transfer the new phosphate group to ADP, thus producing ATP and 3-phosphoglycerate, which is therefore the first substrate level site of ATP synthesis. After rearrangement to 2-phosphoglycerate and dehydration to phosphoenolpyruvic acid, the second phosphate is removed to produce pyruvic acid and ATP, and so is the second site of substrate level ATP synthesis. As mentioned above, depending on the activity of the electron transport chains and

the energy requirements of the cell balanced against the need for certain metabolic intermediates, pyruvate, or its derivatives may now be reduced by accepting the hydrogen from NADH and so continue on a fermentation route or it may be decarboxylated to an acetyl group and enter the TCA cycle. The overall energy balance of glycolysis is discussed later when considering chemical cellular energy production in more detail.

TCA cycle

Pyruvate decarboxylation produces the acetyl group bound to Coenzyme A, ready to enter the TCA cycle otherwise named Kreb's citric acid cycle in tribute to the scientist who discovered it. Not only is this cycle a source of reduced cofactors which 'fuel' electron transport and thus, the synthesis of ATP, but it is also a great meeting point of metabolic pathways. Cycle intermediates are constantly being removed or replenished. During anaerobic fermentation, many of the reactions seen in the TCA cycle are in operation even though they are not linked to electron transport.

Glyoxalate cycle

This is principally the TCA cycle, with two additional steps forming a 'short circuit', involving the formation of glyoxalate from isocitrate. The second reaction requires the addition of acetyl CoA to glyoxalate to produce malic acid and thus rejoin the TCA cycle. The purpose of this shunt is to permit the organism to use acetyl CoA, which is the major breakdown product of fatty acids, as its sole carbon source.

Macromolecules – description and degradation

Lipids

This class of macromolecules (see Figure 2.2) includes the neutral lipids which are triacylglycerols commonly referred to as fats and oils. Triacylglycerols are found in reservoirs in micro-organisms as fat droplets, enclosed within a 'bag', called a vesicle, while in higher animals, there is dedicated adipose tissue, comprising mainly cells full of fat. These various fat stores are plundered when energy is required by the organism as the degradation of triacylglycerols is a highly exergonic reaction and therefore a ready source of cellular energy. Gram for gram, the catabolism of these fats releases much more energy than the catabolism of sugar which explains in part why energy stores are fat rather than sugar. If this were not the case the equivalent space taken up by a sugar to store the same amount of energy would be much greater. In addition, sugar is osmotically active which could present a problem for water relations within a cell, should sugar be the major energy store.

Triacylglycerols comprise a glycerol backbone onto which fatty acids are esterified to each of the three available positions. They are insoluble in an aqueous

environment due to the nonpolar nature of the fatty acids forming 'tails' on the triacylglycerol. However, diacylglycerols and monoacylglycerols which are esterified at only two or one position respectively, may form themselves into micelles due to their polar head, and so may exhibit apparent solubility by forming an emulsion. The tri-, di- and monoacylglycerols have in the past been described as tri-, di- or monoglycerides. Although these are inaccurate descriptions of the chemistry of these compounds the terms tri-, di- and monoglycerides are still in common usage. Chemically, fats and oils are identical. If the compound in question is a liquid at room temperature, frequently it is termed an oil, if solid it is described as a fat. The melting point of these compounds is determined to a large extent by the fatty acid content, where in general, saturated fatty acids, due to their ability to pack together in an orderly manner, confer a higher melting point than unsaturated fatty acids.

Their catabolism is by hydrolysis of the fatty acids from the glycerol backbone, followed by oxidation of the fatty acids by β-oxidation. This process releases glycerol which may then be further degraded by feeding into the central pathways of glycolysis, and several units of the acetyl group attached to the carrier Coenzyme A (Figure 2.2), which may feed into the central metabolic pathways just prior to entry into the TCA cycle (Figure 2.1).

Compound lipids include the phosphoglycerides which are a major component of cell membranes. These can have very bulky polar head groups and nonpolar tails which allow them to act as surfactants and in this specific context, biosurfactants. The most common surfactants are glycolipids (Figure 2.7), which do not have a glycerol backbone, but have sugar molecules forming a polar head and fatty acids forming nonpolar tails, in an overall structure similar to that shown for phospholipids in Figure 2.2. Derived lipids include fat soluble vitamins, natural rubber, cholesterol and steroid hormones. It is interesting to note here that bacteria do not synthesise steroids, and yet some, for example, *Comamonas testosteroni*, are able to degrade specific members of the group; testosterone in the case given (Horinouchi *et al.* 2001). However, oestrogen and its synthetic analogues used in the contraceptive pill, are virtually recalcitrant to decomposition by bacteria. This is proving a problem in waterways especially in Canada where the level of such endocrine disrupters has become so high in some lakes that the feminisation of fish is becoming a concern (McMaster 2001). This subject, and similar more recent findings for the UK, are explored further in Chapter 3.

Proteins

The first catabolic step in protein degradation (see Figure 2.5) is enzymatic hydrolysis of the peptide bond formed during protein synthesis resulting in the release of short pieces, or peptides, and eventually after further degradation, amino acids. The primary step in amino acid catabolism is to remove the amino group thus producing an α-keto acid. This is usually achieved by transfer of the amino group to the TCA cycle intermediate, α-ketoglutarate, resulting in the

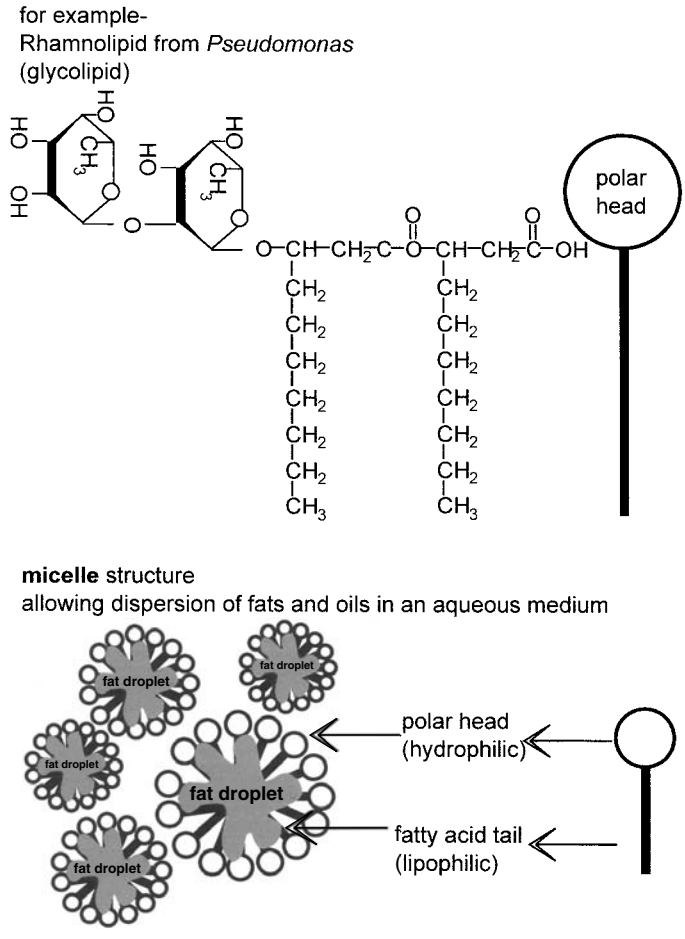


Figure 2.7 *Biosurfactants*

amino acid, glutamate. Amino groups are highly conserved in all organisms due to the small number of organisms able to fix atmospheric nitrogen and so the source of an amino group is usually by transfer from another molecule. However, eventually, nitrogen is removed by oxidative deamination and is excreted in a form which depends upon the organism. Ammonia is toxic to most cells, but if an organism lives in an aqueous habitat, it may release ammonia directly into its surroundings where it is diluted and so made harmless. However, even in such an environment, if dilution should prove insufficient, ammonia concentration will increase, likewise the pH, consequently, the well-being of the organism will be compromised. Organisms which cannot make use of dilution, rid themselves of ammonia by converting it first into a less toxic form such as urea in the case of

mammals and the fairly insoluble uric acid in the case of birds and most reptiles. Bacteria may then convert the excreted ammonia, urea or uric acid into nitrite and then oxidise it to nitrate which may then be taken up by plants. From there it is included in anabolic processes such as amino acid synthesis to produce material ingested by higher animals and the whole procedure of amino group transfer repeats itself. This is the basis of the nitrogen cycle which forms a central part of much of the sewage and effluent treatment described in Chapters 6 and 7.

The α-keto acid resulting from deamination of the amino acid is degraded by a series of reactions, the end product being dependent on the original amino acid, but all will finally result as a glycolysis or TCA cycle intermediate. A fascinating story of catabolism showing collaboration between mammals and bacteria resident in the gut, is the degradation of haemoglobin, the component of blood which carries oxygen and carbon dioxide. Haemoglobin comprises the protein, globin, into which was inserted during synthesis, the haeme ring system where the exchange between binding of oxygen or carbon dioxide takes place in circulating blood. The first step of haemoglobin degradation, performed in the mammalian system, is removal of the haeme ring structure releasing globin which is subject to normal protein degradation. Haeme has its origins in the amino acids in that the starting point for the ring structure is the amino acid, glycine. The degradation pathways starts with removal of iron and release of carbon monoxide to produce the linear structure, bilirubin. This is eventually excreted into the gut where enteric (gut) bacteria degrade the bilirubin to urobilinogens which are degraded further, some being excreted in the urine and others, such as stercobilin, are excreted in the faeces. All these products are further metabolised by microbes, for example, in the sewage treatment plant.

Nucleic acids

Degradation of nucleic acids (see Figure 2.4) is also a source of ammonium ion. The purines are broken down to release CO_2 and uric acid which is reduced to allantoin. This is then hydrolysed to produce urea and glyoxylate which can enter the TCA cycle by the glyoxylate pathway present in plants and bacteria but not mammals. The urea thus produced may be further hydrolysed to ammonium ion or ammonia with the release of carbon dioxide. The form in which the nitrogen derived from the purines is excreted, again depends upon the organism.

Pyrimidines are hydrolysed to produce ammonia which enters the nitrogen cycle, carbon dioxide and β-alanine or β-aminoisobutyric acid both of which are finally degraded to succinyl CoA which enters the TCA cycle.

Carbohydrates

The carbohydrates (see Figure 2.3) form a ready source of energy for most organisms as they lead, by a very short route, into the central metabolic pathways from which energy to fuel metabolic processes is derived. When several sugar units,

such as glucose, are joined together to form macromolecules, they are called polysaccharides. Examples of these are glycogen in animals, and cellulose in plants. In nature, the sugars usually occur as ring structures and many have the general formula, $C(H_2O)_n$, where carbon and water are present in equal proportion. Catabolism of glucose has been described earlier in this chapter. As stated earlier, the resulting metabolite from a given carbon source, or the presence of specific enzymes, can be diagnostic of an organism. Whether or not the enzymes of a particular route are present can help to identify a microbe, and carbohydrate metabolism is frequently the basis of micro-organism identification in a Public Health laboratory. Glucose enters the glycolytic pathway to pyruvate, the remainder of which is determined in part by the energy requirements of the cell and in part by the availability of oxygen. If the organism or cell normally exists in an aerobic environment, there is oxygen available and the pyruvate is not required as a starting point for the synthesis of another molecule, then it is likely to enter the TCA cycle. If no oxygen is available, fermentation, defined later in this chapter, is the likely route. The function of fermentation is to balance the chemical reductions and oxidations performed in the initial stages of glycolysis.

Production of Cellular Energy

Cellular energy is present mainly in the form of ATP and to a lesser extent, GTP (Figure 2.4) which are high energy molecules, so called because a large amount of chemical energy is released on hydrolysis of the phosphate groups. The energy to make these molecules is derived from the catabolism of a food, or from photosynthesis. A food source is commonly carbohydrate, lipid or to a lesser extent, protein but if a compound considered to be a contaminant can enter a catabolic pathway, then it can become a ‘food’ for the organism. This is the basis of bioremediation. The way in which energy is transferred from the ‘food’ molecule to ATP may take two substantially different routes. One is cytoplasmic synthesis of ATP which is the direct transfer of a phosphate group to ADP, storing the energy of that reaction in chemical bonds. The other involves a fairly complicated system involving transfer of electrons and protons, or hydrogen ions, which originated from the oxidation of the ‘food’ at some stage during its passage through the catabolic pathways. The final sink for the electrons and hydrogen ions is oxygen, in the case of oxidative phosphorylation, to produce water. This explains the need for good aeration in many of the processes of environmental biotechnology, where organisms are using oxidative phosphorylation as their main method for synthesising ATP. An example of this is the activated sludge process in sewage treatment. However, many microbes are anaerobes, an example being a class of archaea, the methanogens, which are obligate anaerobes in that they will die if presented with an oxygenated atmosphere. This being the case, they are unable to utilise the oxidative phosphorylation pathways and so instead, operate an electron transport chain similar in principle, although not in detail.

It has as the ultimate electron and hydrogen sink, a variety of simple organic compounds including acetic acid, methanol and carbon dioxide. In this case, the end product is methane in addition to carbon dioxide or water depending on the identity of the electron sink. These are the processes responsible for the production of methane in an anaerobic digester which explains the necessity to exclude air from the process.

Fermentation and respiration

The electrons derived from the catabolism of the carbon source are eventually either donated to an organic molecule in which case the process is described as fermentation, or donated to an inorganic acceptor by transfer along an electron chain. This latter process is respiration and may be aerobic where the terminal electron acceptor is oxygen, or anaerobic where the terminal electron acceptor is other than oxygen such as nitrate, sulphate, carbon dioxide, sulphur or ferric ion. Unfortunately, respiration is a term which has more than one definition. It may also be used to describe a subset of the respiration processes mentioned above to include only oxidation of organic material and where the ultimate electron acceptor is molecular oxygen. This latter definition is the basis of biological oxygen demand (BOD), which is often used to characterise potential environmental pollutants, especially effluents, being a measure of the biodegradable material available for oxidation by microbes.

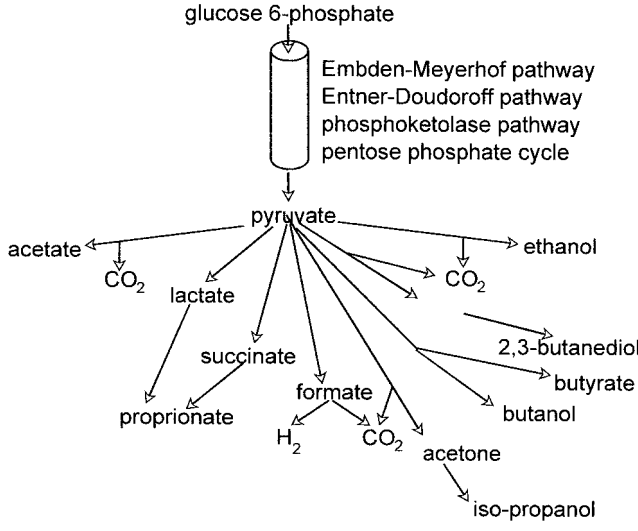
Fermentations

In modern parlance, there are many definitions of the term 'fermentation'. They range from the broadest and somewhat archaic to mean any large-scale culture of micro-organisms, to the very specific, meaning growth on an organic substance and which is wholly dependent on substrate-level phosphorylation. This is the synthesis of ATP by transfer of a phosphate group directly from a high energy compound and not involving an electron transport chain. Additionally, and a source of great confusion, is that fermentation may refer simply to any microbial growth in the absence of oxygen but equally may be used generally to mean microbial growth such as food spoilage where the presence or absence of oxygen is unspecified. The definition used throughout this book, except with reference to eutrophic fermentation discussed in Chapter 8, is that of growth dependent on substrate-level phosphorylation.

There are very many fermentation routes but all share two requirements, the first being the regeneration of NAD^+ from NADH produced during glycolysis which is essential to maintain the overall reduction: oxidation equilibrium, and the second being that pyruvate, or a derivative thereof, is the electron acceptor during the reoxidation of NADH. What this means is that all fermentation routes start with pyruvate, the end-point of glycolysis, and proceed along a variety of pathways to an end product indicative, if not diagnostic, of the organism.

Fermentation is therefore an option under conditions where there is an active electron transport chain as discussed in the following section, but becomes essential when fermentation is the only method for regenerating NAD^+.

As noted above, the end product of fermentation for any given carbon source may be diagnostic of the identity of a specific organism. This is more relevant for bacteria than for yeast or other eukaryotic cells and arises from the predisposition of that organism, to use a particular fermentation pathway. These are described in detail in Mandelstam and McQuillen (1973) and are summarised in Figure 2.8. Identification by the product of carbohydrate catabolism is somewhat specialised and is very thoroughly set out in *Cowan and Steel's Manual for the Identification of Medical Bacteria* (Barrow and Feltham 1993).



examples of end products of pyruvate fermentation typically shown by the organisms listed

acetate	Enterobacteria, Clostridia
acetoin	yeast
acetone	Clostridia
2,3-butanediol	yeast, Aerobacter
butanol	Clostridia
butyrate	Clostridia
ethanol	yeast, acetobacter, enterobacteria
formate	Enterobacteria
iso-propanol	Clostridia
lactate	lactic acid bacteria (*Streptococcus, Lactobacillus*)
proprionate	*Clostridium proprionicum*

Figure 2.8 *Fermentations*

Electron transport chains: oxidative phosphorylation and methanogenesis

As described in the previous section, NADH and other reduced cofactors may be reoxidised by the reduction of organic receptors such as pyruvate. This is the fermentation route.

Alternatively, the reducing agent (or reductant) can transfer the electrons to an electron transport chain which ultimately donates them to an inorganic receptor (the oxidising agent or oxidant). In aerobic respiration, this receptor is oxygen. However, some bacteria have electron transport chains which use other electron sinks such as nitrate, sulphate, carbon dioxide and some metals, with respiration being described as anaerobic in these cases. The use of nitrate in this role leads to the process of denitrification, which plays an important part in many aspects of the applications of environmental biotechnology.

A number of events occur during the flow of electrons along the chain which have been observed and clearly described for a number of organisms and organelles, most especially the mitochondria of eukaryotic cells. These are fully discussed in many biochemistry textbooks, an excellent example being Lehninger (1975), the gist of which is outlined in this section. The details of exactly how these phenomena combine to drive the synthesis of ATP is still unclear but various models have been proposed.

The chemiosmotic model, proposed by Peter Mitchell in 1961, states that the proton, or hydrogen ion, gradient which develops across an intact membrane during biological oxidations is the energy store for the subsequent synthesis of ATP. This model somewhat revolutionised the then current thinking on the energy source for many cellular processes, as the principles of energy storage and availability according to the chemiosmotic theory were applicable to many energy-demanding cellular phenomena including photosynthetic phosphorylation and some cross-membrane transport systems. It could even account for the movement of flagellae which propel those bacteria possessing them, through a liquid medium. The chemiosmotic theory accounts for the coupling of the transmembrane proton gradient to ATP synthesis. It implies that during oxidation, the electrons flow down from high to low energy using that energy to drive protons across a membrane against a high concentration, thus developing the proton gradient. When the electron flow stops, the protons migrate down the concentration gradient, simultaneously releasing energy to drive the synthesis of ATP through membrane-associated proteins. The model system described first is that of mitochondria and, later in this chapter, comparisons with bacterial systems associated with oxidative phosphorylation and those systems associated with methanogenesis will be made.

Electron transport chains comprise cytochrome molecules which trap electrons, and enzymes which transfer electrons from a cytochrome to its neighbour. The quantity of energy released during this transfer is sufficient to drive the synthesis of approximately one ATP molecule by the enzyme *ATP synthetase*. The whole system is located in a membrane which is an essential requirement of any electron

transport chain because of the need to organise it topographically, and to allow the establishment of a pH gradient. Also there is evidence that during active electron transport, the morphology of the membrane changes and is believed to store energy in some way yet to be elucidated. Consequently, an intact membrane is essential. Any toxic substance which damages the integrity of a membrane has the potential to interrupt the functioning of the electron transport chain thereby reducing the facility for ATP synthesis and potentially killing the organism. The chain may also be disrupted by interference with the electron carriers. Such a chemical is cyanide, which complexes with cytochrome oxidase, and for which research into a biological remediation route is underway.

The mitochondrial electron transport system and oxidative phosphorylation

The electron transport system in eukaryotes is located in the inner membrane of mitochondria. A representation of the system is given in Figure 2.9. The chain is a series of complexes comprising cytochromes, and enzymes involved in oxidation–reduction reactions whose function is to transfer electrons from one complex to the next. The ratios of the complexes one to another varies from cell type to cell type. However, the concentration of the *cytochrome a* complex per unit area of inner membrane stays fairly constant. What changes from cell type to cell type is the degree of infolding of the inner membrane, such that cells requiring a large amount of energy have mitochondria which have a very large surface area of inner membrane, which is highly convoluted thus providing a high capacity for electron transport. The process which couples ATP synthesis to electron transport in mitochondria and which still evades a complete description, is oxidative phosphorylation or more accurately, respiratory-chain phosphorylation. There are three sites within the mitochondrial chain which span the interaction between two neighbouring complexes, which on the basis of energy calculations are thought to witness a release of energy sufficient to synthesise almost one molecule of

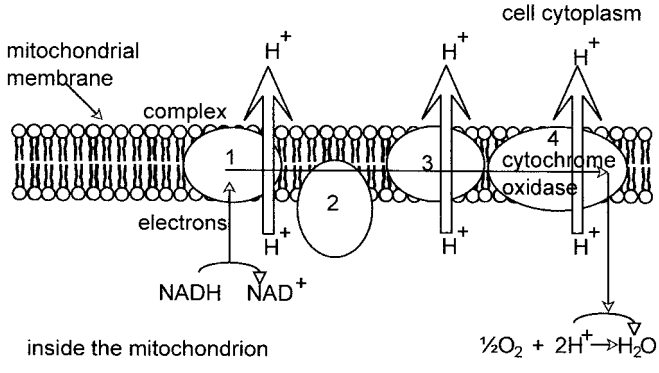


Figure 2.9 *Mitochondrial electron transport chain*

ATP from ADP and phosphate, as a result of electron transfer from one complex to its neighbour. These are designated site I between NADH and coenzyme Q, site II between *cytochromes b* and *c*, and site III between *cytochrome a* and free oxygen. Site III occurs within complex IV, the final complex which may also be referred to as *cytochrome oxidase*. Its overall function is to transfer electrons from cytochrome c to cytochrome a, then to a_3 and finally to molecular oxygen. It is this final stage which is blocked by the action of cyanide and by carbon monoxide. Associated with the electron flow, is the ejection of hydrogen ions from inside the mitochondrion, across the membrane, and in complex IV, the reduction of the oxygen molecule with two hydrogen ions originating from inside the mitochondrion. If all three sites were involved, the amount of energy released is sufficient to drive the synthesis of two and a half molecules of ATP for each pair of electrons transported. If the first site was omitted, the number falls to one and a half. In neither case is it a complete integer because there is not a direct mole for mole relationship between electron transport and ATP synthesis but as described earlier, it is part of a much more complicated process described above as the chemiosmotic theory.

Bacterial electron transport systems and oxidative phosphorylation

Bacterial electron transport chains have fundamentally the same function as that described for mitochondrial electron transport chains but with several notable differences in their structure. For example, the cytochrome oxidase which is the final complex nearest the oxygen in mitochondria, is not present in all bacteria. The presence or absence of this complex is the basis of the 'oxidase' test for the identification of bacteria. In these organisms, cytochrome oxidase is replaced by a different set of cytochromes. An interesting example is *Escherichia coli*, an enteric bacterium and coliform, which is commonly found in sewage. It has replaced the electron carriers of cytochrome oxidase with a different set including cytochromes b_{558}, b_{595}, b_{562}, d and o, which are organised in response to the level of oxygen in the local environment. Unlike the mitochondrial chain, the bacterial systems may be highly branched and may have many more points for the entry of electrons into the chain and exit of electrons to the final electron acceptor.

Bacterial electron transport systems, denitrification and methanogenesis

As previously mentioned, the term respiration is applied to many processes. Without further specification it is usually used to mean the consumption of molecular oxygen, by reduction to water in the case of the electron transport discussed above, or by oxidation of an organic molecule to produce carbon dioxide and serine in the case of photorespiration, discussed later in this chapter. Thus the term anaerobic respiration seems a contradiction. It does, however, describe fundamentally the same process of electron transfer to a final acceptor which although inorganic, in this case is not oxygen. An example of such an electron acceptor

is nitrate which is converted to nitrite. This is a toxic substance, and so many bacteria have the facility to convert nitrite to nitrogen gas. This overall series of reactions is described as denitrification and is the basis of the process by which denitrifying bacteria such as members of the *Pseudomonas* and *Bacillus* genera are able to reduce nitrate and nitrite levels down to consent values during sewage treatment. Such bacteria have different components in their electron transport chain in comparison with mitochondria, which have the necessary enzymatic activities to carry out these processes. Like mitochondrial electron transport, denitrification can be associated with synthesis of ATP although with much reduced efficiency.

Other examples of terminal electron acceptors are firstly sulphate, in which case one of the final products is elemental sulphur. This process is carried out by the obligate anaerobe, *Desulfovibrio* and members of the archaean genus *Archaeglobus.* Another anaerobe, *Alkaliphilus transvaalensis,* an extreme alkaliphile, growing at a pH of 8.5 to 12.5, isolated from an ultra-deep gold mine in South Africa, can use elemental sulphur, thiosulphate or fumarate as an additional electron acceptor (Takai *et al*. 2001). Secondly, carbon dioxide may be the final electron acceptor in which case one of the final products is methane. This process is also carried out by obligate anaerobes, in this case, the methanogens, all of which are archaeans and are responsible for methane production in anaerobic digesters and landfill sites. Again, it functions on much the same principles as the other chains mentioned above but has a different set of cofactors which are most unusual. For both of the above obligate anaerobes, anaerobic respiration is an important mechanism of ATP synthesis. It is less efficient than aerobic respiration due to the smaller drop in electropotential between sulphate or carbon dioxide and NADH compared with the difference between NADH and oxygen, and so less energy is available to be released during electron transport and consequently less ATP is synthesised per mole of NADH entering the pathway. Anaerobic respiration is, however, more efficient than fermentation and so is the route of choice for ATP synthesis for an anaerobe.

The energy balance sheet between substrate level and electron transport linked ATP synthesis

An approximate comparison may be made between the efficiency with respect to energy production, of ATP synthesis by substrate-level phosphorylation and by association with electron transport. For one mole of glucose passing through glycolysis by the Embden–Meyerhof pathway to produce two moles of pyruvate, there is net production of two moles of ATP. For most fermentation pathways, no further ATP is synthesised. There are exceptions, of course, such as the conversion of an acyl CoA derivative such as acetyl CoA or butyryl CoA to the free acid which in these cases are acetate and butyrate respectively. Each of these reactions releases sufficient energy to drive the phosphorylation of one mole of ADP. Conversely, if the electron transport chain is functioning, NADH may be

oxidised by relinquishing electrons to the cytochromes in the chain thus regenerating the oxidised cofactor. In this scenario, pyruvate may enter the TCA cycle rather than a fermentation route, thus a further mole of ATP is produced at substrate level during conversion of succinyl CoA to succinate via GTP, which then transfers the terminal phosphate to ATP. In addition, NADH and $FADH_2$ are produced during the TCA cycle thus generating up to 15 moles of ATP per mole of pyruvate. An overall comparison may be made between glycolysis followed by reoxidation of NADH by fermentation or, alternatively, glycolysis followed by entry into the TCA cycle and reoxidation of cofactors via the electron transport chain. Remembering that one mole of glucose generates two moles of pyruvate during glycolysis, and that the two moles of NADH produced during glycolysis may also be reoxidised by transfer to the electron transport chain and not through fermentation, the net result is that glucose catabolised by the glycolysis–fermentation route results in the production of two moles of ATP whereas catabolism by the glycolysis–TCA cycle–electron transport/oxidative phosphorylation route produces up to 32 moles of ATP. The figure of 36 was deduced by Lehninger (1975) but has been revised more recently to reflect the tenets of the chemiosmotic theory described earlier.

Anaerobic respiration is less efficient than aerobic respiration. Oxidation of the same amount of cofactor by methanogenesis rather than oxidative phosphorylation would produce fewer moles of ATP. Consequently, for a given amount of ATP production, the flux of glucose through glycolysis followed by fermentation would have to be approximately 16 times greater than through glycolysis followed by oxidative phosphorylation, and the flux through methanogenesis is somewhat intermediate. It is the metabolic capability of the organism and the presence or absence of the appropriate inorganic electron acceptor which determines the fate of pyruvate on the grounds of energy considerations. On a practical basis this may explain why anaerobic processes, such as the anaerobic digestion of sewage sludge and municipal solid waste, are considerably less exothermic than their aerobic counterparts. For a given quantity of carbon source, an aerobic process will be able to extract in the order of 10 times the amount of energy than that generated by an anaerobic process.

Regeneration of NAD^+ in plants

In addition to the processes discussed above for the production of NADH, plant mitochondria operate an additional system whereby the required protons are derived from two molecules of the amino acid glycine. During this mitochondrial process, one molecule of molecular oxygen is consumed in the production of carbon dioxide and the amino acid, serine. The superfluous amino group from the second glycine molecule is released as ammonia. The glycine molecules were derived from phosphoglycolate, the metabolically useless product of photorespiration. This subject is very important with regard to plant breeding and

development and so is discussed in some detail alongside the related subject of photosynthesis.

Photosynthesis and the Basis of Phytotechnology

The sun is the biosphere's ultimate source of energy and photosynthesis is the only means there is on this planet to trap incident sunlight and convert it into chemical energy available to biological processes. Thus, with very rare exceptions, organisms which do not photosynthesise, which is the majority, are totally dependent on those which do. With this introduction it is hardly surprising to find a description of this process in a book which specifically addresses the capabilities of biological organisms and their interplay. Leafy plants obviously feature in this section but so too do photosynthetic eukaryotic micro-organisms and bacteria. A knowledge of this vital process is essential to appreciating the role which photosynthesising organisms play in the environment, their limitations and the strengths upon which biotechnology can capitalise.

The energy from this process is used to drive all the biochemical synthesis and degradation reactions occurring in the cell in addition to various other energy-requiring processes such as the movement and transport of molecules across membranes. Energy is finally dissipated as heat, and entropy rises in accordance with the laws of thermodynamics. Any interference with the flow from the sun either by reducing the ability of the energy to penetrate the atmosphere, or by reducing the total photosynthetic capacity of the planet, has dramatic consequences to all forms of life. Conversely, too intense a radiation from the sun resulting from thinning of the ozone layer runs the risk of damaging the photosynthetic machinery. This can be compensated for by the organism acquiring pigments to absorb harmful radiation, but this requires time for such an evolutionary adjustment to take place.

It is noteworthy that the bulk of photosynthesis is performed by unicellular organisms, such as photosynthetic algae, rather than the macrophytes as might reasonably be supposed. Photosynthesis occurs in two parts; the first is the trapping of light with associated reduction of $NADP^+$ and ATP synthesis, and the second is the fixing of carbon dioxide by its incorporation into a carbohydrate molecule. This is most commonly a hexose sugar, and typically glucose, the synthesis of which utilises the NADPH and ATP produced in the light-dependent part 1. The processes of carbohydrate synthesis occurring in the second part are described as the dark reactions, so called because they may proceed in the dark after a period of illumination to activate part 1. The sugar produced during these dark reactions will then be utilised by the cell, transferred to another cell or ingested by a larger organism and eventually catabolised to carbon dioxide and water, releasing the energy consumed originally to synthesise the molecule. Here is another example of a natural cycle, where carbon is introduced, as carbon dioxide, into the synthesis of a sugar which is then interconverted through the various metabolic pathways until finally it is released as carbon dioxide thus completing

the cycle. Eukaryotes capable of carrying out photosynthesis include higher green plants, multicellular green, brown and red algae and various unicellular organisms such as the euglenoids and dinoflagellates both of which are commonly found in fresh water environments, and diatoms which are also found in salt water. The diatoms which are unicellular algae, are particularly noteworthy given the current estimates that they are responsible for fixing 20 to 25% of the world's carbon through photosynthesis (Round, Crawford and Mann 1990). Prokaryotes capable of photosynthesis include blue-green algae, and both the sulphur and nonsulphur purple and green bacteria. The blue-green algae which are oxygenic bacteria and are alternatively named cyanobacteria, operate light reactions very similar to those of eukaryotes. Conversely, the green and the purple nonsulphur bacteria which are both facultative aerobes and the strictly anaerobic green and the purple sulphur bacteria utilise a rather different set of light reactions as a consequence of their possessing a 'simpler' photosystem. Eukaryotic and bacterial systems are both described in the following sections.

The light reactions

Visible light is the outcome of the nuclear fusion of hydrogen atoms, resulting in the production of helium atoms, gamma radiation and two electrons. This fusion occurs in the sun at a temperature of approximately 20 000 000 K. The gamma radiation and electrons combine to produce quanta of visible light. The entrapment of light is performed in photosynthetic cells by pigments; the most important of which are the chlorophylls. These are flat ring structures, with regions of conjugated double and single bonds, and a long hydrophobic tail well designed for anchoring the pigments into membrane. Only red and blue light is absorbed by the chlorophylls in most organisms. Consequently, when white light from the sun shines upon them, they reflect green light, thus making these organisms appear green. Variation in the types of chlorophylls and the presence of additional accessory pigments all contribute to the observed colour of the organism and are the result of evolution which has developed the 'best fit' of light-trapping molecules to suit the ecological niche of the organism. It is worth pointing out that wholesale transport of the plant or bacterium for biotechnology purposes has to take this factor into account. It is important to test the growth and performance characteristics of any translocated plant or bacterium to ensure that the new environment does not produce disappointing results. This problem is addressed with respect to choice of *Phragmites* species in the case study on reed beds in Chapter 7. The purpose of the accessory pigments referred to above, which include the carotenoids and phycobiliproteins, the latter found in red algae and cyanobacteria, is to extend the range of absorbed wavelengths thus maximising the amount of energy trapped from light and protecting the photosynthetic system from potential damage by oxidation. A rather unusual pigment which functions as a primary pigment, is bacteriorhodopsin which causes the archaea which express it, to appear purple. Returning to the principally eukaryotic process, the chlorophylls described above

which receive the incident light are clustered in highly organised structures called antennae located on the cell surface. The incident light excites the energy state of the recipient chlorophyll to a higher energy state. When the chlorophyll returns to its normal level, it releases electrons which are transferred to a neighbouring chlorophyll. The transfer is repeated until the electrons arrive at a photosystem, from which point they enter an electron transport chain linked to the reduction of NAD^+ and the synthesis of ATP. There are many similarities between electron transport in respiration and in photosynthesis in that they are both membrane bound and may be coupled to phosphorylation and thus synthesis of ATP, according to Mitchell's chemiosmotic theory, by employing a similar strategy of a proton gradient described earlier for respiration. In eukaryotic higher organisms, photosynthesis occurs in the chloroplast while in bacteria it occurs in the cytoplasmic membrane. The precise location in bacteria is sometimes described as being the mesosome. This has been reported as an infolding of the bacterial cellular membrane which sometimes appears to be in association with the bacterial DNA and often is found near nascent cell walls. Although considerable effort has been invested in determining its function, there is still disagreement as to whether or not the mesosome is indeed a bacterial cell structure or is simply an artefact occurring during preparation of samples for microscopy. Thus the site of bacterial photosynthesis remains uncertain beyond it being bound in the cytoplasmic membrane.

Photosystems in eukaryotes and cyanobacteria

There are two types of photosystem which may occur in photosynthetic organisms indicated in Figure 2.10: photosystem 1 which receives electrons from photosystem 2 but may also operate independently by cyclic electron transport, and photosystem 2 which is not present in all such organisms. The pathway for electron transport has two principal routes. One involves only photosystem 1. In this, electrons transferred from the antennae to photosystem 1 cause excitation of the chlorophyll in this system. When the chlorophyll returns to its lower energy state, the electrons are transferred to ferredoxin, which is one of the iron-containing proteins in the chain of electron carriers. From this point there is a choice of routes; either the cyclic path by way of a chain of cytochrome molecules starting with cytochrome b_{563} and finally returning to chlorophyll a, or by the noncyclic route which is the transfer of electrons to $NADP^+$.

The source of the hydrogen atom required to reduce the $NADP^+$ to NADPH in this system is the water molecule which donates its electrons to photosystem 2 to replace those lost to $NADP^+$. It is the origin of the oxygen released during photosynthesis, hence the term oxygenic. Thus the overall flow of electrons in the noncyclic pathway is from the water molecule, through photosystem 2, along a series of cytochromes to photosystem 1 and thence to ferredoxin and finally $NADP^+$, which also collects a hydrogen atom to complete the reduction

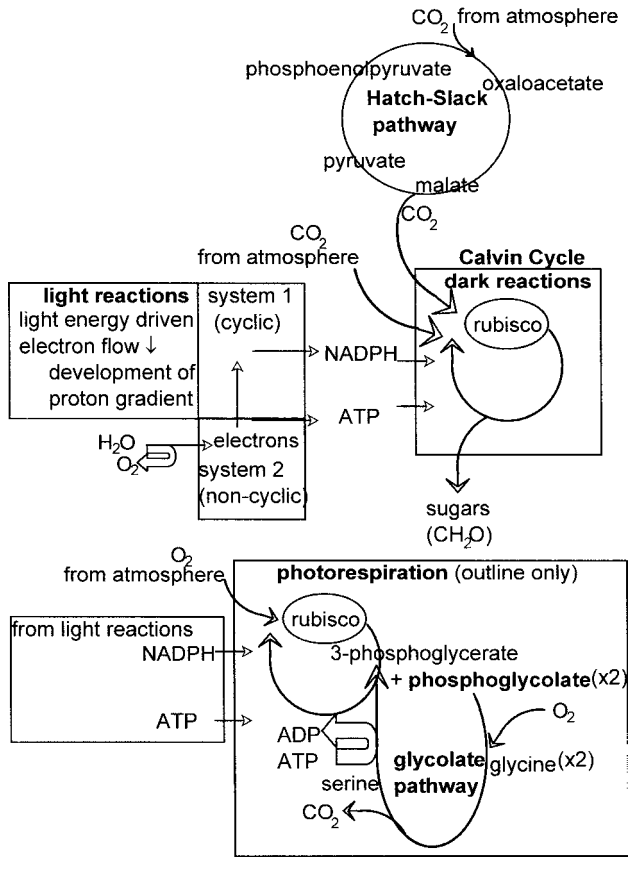


Figure 2.10 *Oxygenic photosynthesis, photorespiration and the Hatch–Slack pathway*

to NADPH. Both the cyclic and the noncyclic pathways produce a proton gradient which drives the synthesis of ATP, but only the cyclic route has the facility of producing NADPH. A combination of cyclic and noncyclic pathways are used by the organism to produce the required amounts of NADPH and ATP used in the dark reactions for the synthesis of carbohydrate. So far the description has been of photosynthesis in eukaryotes and cyanobacteria.

Photosystems in purple and green bacteria

Looking at the general equation for the chemical reactions in photosynthesis shown in Figure 2.11, it may be seen that water is the electron donor during oxygenic photosynthesis while several molecules may play the same role in anoxygenic systems. Suitable molecules are listed in the figure, from which several interesting observations may be made. If the electron donor is hydrogen

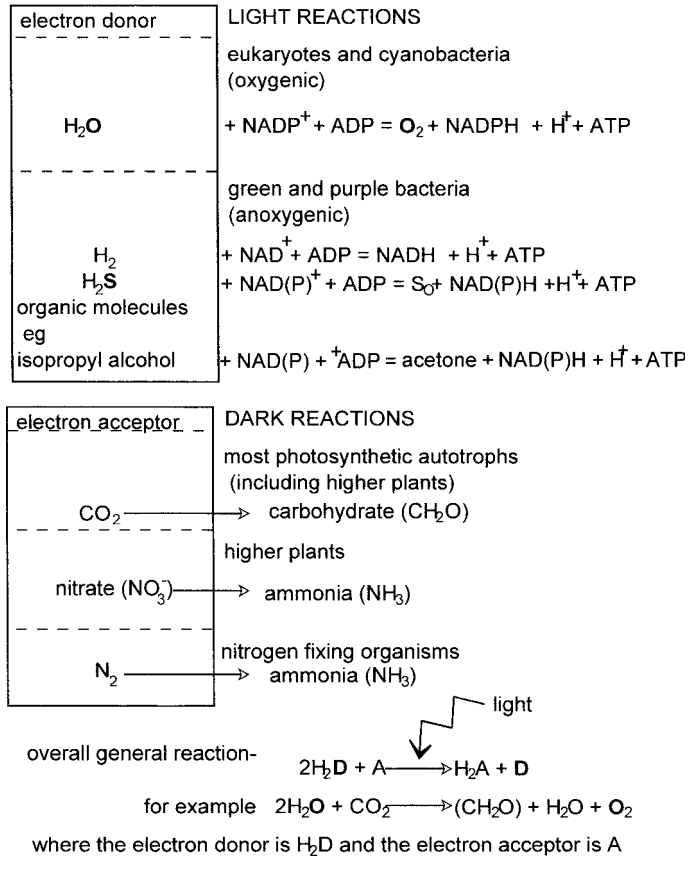


Figure 2.11 *Reactions of photosynthesis*

sulphide, which is the principal gas responsible for the foul smell, reminiscent of rotten eggs, typically found in wet and untilled soil, for example in the bottom of ponds, the product is sulphate or elemental sulphur. Examination of bacterial photosystems explains how this occurs.

Green and purple bacteria possess only one photosystem which is a fairly basic equivalent to photosystem 1 of eukaryotes and cyanobacteria, but employing a different set of electron carriers. In purple nonsulphur bacteria, this is only capable of cyclic electron flow which produces a proton gradient and thus allows ATP photosynthesis, but this process does not lead to NADPH production. A similar system exists in green nonsulphur bacteria. The lack of a photosystem equivalent to photosystem 2 in eukaryotes, requires these bacteria to provide a different route for the regeneration of NADH which serves much the same function as NADPH in carbohydrate synthesis. Their solution to this problem is to use as

electron donors, molecules which have a more negative reduction potential than water and so are easier to oxidise. These include hydrogen, hydrogen sulphide, elemental sulphur and a variety of organic compounds including sugars, and various organic acids such as amino acids, and succinate.

There are many ways in which green and purple nonsulphur bacteria may produce NADH. For example, direct reduction is possible if they are growing in the presence of dissolved hydrogen gas due to the fact that hydrogen has a more negative reduction potential than NAD^+. In addition, purple nonsulphur photosynthetic bacteria may use ATP or the proton gradient established during photosynthesis to reverse the electron flow such that the direction is from one of the electron donors noted above to NAD^+.

Green and purple sulphur bacteria are rather different in that in addition to having a cyclic system broadly similar to that of purple bacteria, they have an additional enzyme activity which allows the nonlinear transfer of electrons to ferredoxin linked to NAD^+ resulting in the production of NADH. One of the sources of electrons to replace those used in this reduction is the oxidation of hydrogen sulphide to sulphate or elemental sulphur, in a process comparable to the oxidation of water in oxygenic organisms. Other electron donors which may be used in this way are hydrogen and elemental sulphur. Both of these nonsulphur bacteria are strict anaerobes.

Photosystem in a halophile

A photosystem which is different again from those described so far is that found in the halophile *Halobacterium salinarium* which has previously been classified as *Halobacterium halobium.* Under normal conditions this organism obtains its energy by respiration, but in order to survive conditions of low oxygen concentrations, it can photosynthesise provided there is sufficient light. The pigment which has been developed for this purpose is bacteriorhodopsin, which is very similar to the rhodopsin pigment found in vertebrate eyes. The part of the molecule which absorbs light is retinal. When this occurs, changes in the bond formation of this chromophore result in expulsion of protons across the membrane thus producing a proton gradient. As described for other systems, this proton gradient may then be used to drive ATP synthesis.

The dark reactions

The result of illumination of a photosynthetic organism is to stimulate electron transport which leads to the production of NADPH or NADH, and synthesis of ATP. Both are required for the next stage which in eukaryotes and cyanobacteria (blue-green algae) is the synthesis of sugar from carbon dioxide involving the Calvin cycle. Many biochemistry textbooks give excellent descriptions of this process and so only a summary is given in Figure 2.10.

In brief, ribulose diphosphate is carboxylated with carbon dioxide catalysed by the enzyme rubisco to form an unstable six-carbon sugar which is then cleaved

to form two molecules of 3-phosphoglycerate, an intermediate of glycolysis. This is not the only route of entry of carbon dioxide into carbohydrate synthesis, the other being the Hatch–Slack pathway. This subject is discussed in more detail later in this chapter.

Returning to the Calvin cycle, rearrangements of 3-phosphoglycerate produced by rubisco then take place by similar steps to the reversible steps of glycolysis and reactions of the pentose phosphate pathway. After completion of three cycles, the net result is the fixation of three molecules of carbon dioxide into a three-carbon sugar, each cycle regenerating a ribulose phosphate molecule. After phosphorylation of the trioses at the expense of ATP, they may enter into glycolysis and be converted into glucose and then to starch to be stored until required. The Calvin cycle is so familiar that it is easy to overlook the fact that not all reducing equivalents are channelled through rubisco to this cycle and carbohydrate synthesis.

Some organisms may use additional pathways involving other electron acceptors such as nitrate, nitrogen and hydrogen atoms, the reduction of which obviously does not produce carbohydrate but different essential nutrients which may then also be available to other organisms. These products are summarised in Figure 2.11. For example, when nitrogen or nitrate is the electron donor, the product is ammonia which becomes incorporated by amino transfer into amino acids and thus forms part of the nitrogen cycle. Nitrogen is a particularly noteworthy case in the context of this book as it is the process of nitrogen fixation. This is performed by a number of nitrogen-fixing bacteria some of which are free living in the soil and some form symbiotic relationships with certain leguminous plants, forming root nodules. Nitrogen fixation is, by necessity, an anaerobic process, and so one essential role for the plant is to provide a suitable oxygen-free environment for these bacteria, the other, to provide energy. Genetic manipulation of plants is discussed in Chapter 9 but it is relevant to point out here that the suggestion is often mooted of introducing the genes responsible for allowing nitrogen fixation to be transferred from the relevant bacteria into suitable plants. The symbiotic relationship between plant and bacteria is very difficult to create artificially and has been a stumbling block in the drive to increase the number of plant species able to host nitrogen fixation. The complicated interaction between plant and bacterium involves intricacies of plant physiology as well as genetic capability provided by the bacterium, and so it is unlikely that a simple transfer of nitrogen fixation genes from bacterium to plant will be successful. This, however, remains a research area of major importance.

The issue of nitrogenous material, particularly in respect of sewage and associated effluents, is of considerable relevance to the environmental application of biotechnology. In addition, there is great potential for phytotechnological intervention to control nitrogen migration, most especially in the light of the burgeoning expansion of nitrate-sensitive areas within the context of agricultural fertiliser usage. Hence, bioengineering of the nitrogen cycle, at least at the local

level, provides an important avenue for the control of pollution and the mitigation of possible eutrophication of aquatic environments. The cycle itself, and some of the implications arising, are discussed later in this chapter.

C_3 and C_4 plants

Plants for which the reaction catalysed by rubisco is the first point of entry of atmospheric carbon dioxide into carbohydrate metabolism are termed C_3 plants due to the product of rubisco being two molecules of 3-phosphoglycerate which contains three carbons. This is the typical route for temperate organisms. An alternative to direct carboxylation for introducing carbon dioxide into the Calvin cycle used by some tropical plants, is the Hatch–Slack pathway illustrated in Figure 2.10. In this case the first step of entry for atmospheric carbon dioxide is by carboxylation of phosphoenolpyruvate by *phosphoenolpyruvate carboxylase* to produce the four-carbon molecule, oxaloacetate. Hence, plants able to use this pathway are termed C_4 plants. The oxaloacetate is part of a cycle which carries the carbon dioxide into the bundle-sheath cells and so away from the surface of the plant, to where the oxygen concentration is lower. Here the carbon dioxide, now being carried as part of malate is transferred to rubisco thus releasing pyruvate which returns to the mesophyll cells at the surface of the plant where it is phosphorylated at the expense of ATP to phosphoenolpyruvate ready to receive the next incoming carbon dioxide molecule from the atmosphere.

The overall effect is to fix atmospheric carbon dioxide, transfer it to a site of lower oxygen concentration compared with the surface of the plant, concentrate it in the form of malate and then transfer the same molecule to rubisco where it enters the Calvin cycle. Although the Hatch–Slack pathway uses energy, and therefore may seem wasteful, it is of great benefit to plants growing in the warmer regions of the globe The reason for this is that the enzyme involved in carbon dioxide fixation in C_4 plants namely *phosphoenolpyruvate carboxylase* has a very high affinity for carbon dioxide and does not use oxygen as a substrate, contrasting with rubisco. The result of this competition between oxygen and carbon dioxide for binding to rubisco is the futile process of photorespiration, described in the next section. The affinity of carbon dioxide for rubisco falls off with increasing temperature and so in a tropical environment, the efficiency of rubisco to fix carbon dioxide is low. In this situation, the disadvantage in using energy to operate the Hatch–Slack pathway is more than compensated for by the advantage of being able to fix carbon dioxide efficiently at elevated temperatures. So advantageous is this that much research is being directed to transferring the capability to operate the Hatch–Slack pathway into selected C_3 plants.

In the broadest sense of environmental biotechnology, the potential maximisation of solar energy usage, either as a means to the remediation of contamination or to reduce potential pollution by, for example, excessive fertiliser demand, could be of considerable advantage. Hence, appropriately engineered C_3 plants

in either role offer major advantages in solar efficiency, which, in temperate climes, could provide significant environmental benefits.

Photorespiration

Returning to synthesis of carbohydrate by the Calvin cycle, as mentioned above, the first step is the carboxylation of the five-carbon sugar, ribulose diphosphate catalysed by rubisco. As mentioned in the preceding section, this enzyme may also function as an *oxidase* indicated by its full name *ribulose diphosphate carboxylase oxidase*. When this occurs and oxygen replaces carbon dioxide, the ensuing reaction produces phosphoglycolate in addition to 3-phosphoglycerate. Since, as a result of illumination, oxygen is consumed and carbon dioxide is released during the reactions of the glycolate pathway, this process is termed photorespiration and occurs alongside photosynthesis. The higher the ambient temperature in which the organism is growing, and the higher the oxygen concentration relative to carbon dioxide, the more pronounced the *oxidase* activity becomes and consequently the less efficient rubisco is at introducing carbon dioxide into carbohydrate synthesis. The phosphoglycolate formed as a result of oxygen acting as substrate for rubisco, is then dephosphorylated to form glycolic acid. There follows a series of reactions forming a salvage pathway for the carbons of glycolic acid involving transfer of the carbon skeleton to the peroxisomes, then to the mitochondria, back to the peroxisomes and finally back to the chloroplast in the form of glycerate which is then phosphorylated at the expense of ATP to re-enter the Calvin cycle as 3-phosphoglycerate. The result of this detour, thanks to the *oxidase* activity of rubisco is to lose a high energy bond in phosphoglycolate, to consume ATP during phosphorylation to produce 3-phosphoglycerate, consumption of oxygen and release of carbon dioxide. This pathway resulting from the *oxidase* activity of rubisco, shown in Figure 2.10 is wasteful because it consumes energy obtained by the light reactions with no concomitant fixation of carbon dioxide into carbohydrate. The C_3 plants are therefore operating photosynthesis under suboptimal conditions especially when the oxygen tension is high and carbon dioxide tension is low. Why rubisco has not evolved to lose the *oxidase* activity is unclear: presumably evolutionary pressures of competition have been insufficient to date. For the reasons indicated in the preceding section, C_4 plants show little or no photorespiration due to their ability to channel carbon dioxide to rubisco by a method independent of oxygen tension. Therefore they are considerably more efficient than their C_3 counterparts and may operate photosynthesis at much lower concentrations of carbon dioxide and higher concentrations of oxygen. It is interesting to contemplate the competitive effects of introducing C_4 style efficiency into C_3 plants, but at the moment this is just speculation.

Balancing the light and the dark reactions in eukaryotes and cyanobacteria

Using the six-carbon sugar, glucose, as an example, synthesis of one molecule requires six carbon dioxide molecules, 12 molecules of water, 12 protons,

18 molecules of ATP and 12 molecules of NADPH. Since photophosphorylation is driven by a proton gradient established during electron flow after illumination, there is not a stoichiometric relationship between the number of photons exciting the systems and the amount of ATP produced. However, it is now established that for every eight photons incident on the two photosystems, four for each system, one molecule of oxygen is released, two molecules of $NADP^+$ are reduced to NADPH and approximately three molecules of ATP are synthesised. Since this may leave the dark reactions slightly short of ATP for carbohydrate synthesis, it is postulated that photosystem 2 passes through one extra cycle thus producing additional ATP molecules with no additional NADPH.

The Nitrogen Cycle

Nitrogen is constantly taken, or fixed, from the atmosphere, oxidised to a form able to be utilised by plants and some bacteria, to be subsumed into metabolic pathways, and through the various routes described above is then excreted into the environment as reduced nitrogen where it may be reoxidised by bacteria or released back into the atmosphere as nitrogen gas. These combined processes are known collectively as the nitrogen cycle. The previous discussions have referred to the release of nitrogen during degradation of proteins and nucleic acid bases, either in the form of ammonia, the ammonium ion, urea or uric acid. The fate of all these nitrogen species is to be oxidised to nitrite ion by *Nitrosomas*, a family of nitrifying bacteria. The nitrite ion may be reduced and released as atmospheric nitrogen, or further oxidised to nitrate by a different group of nitrifying bacteria, *Nitrobacter.* The process of conversion from ammonia to nitrate is sometimes found as a tertiary treatment in sewage works to enable the nitrate consent to be reached. The process typically occurs in trickling bed filters which have, over time, become populated with a *Nitrosomas* and *Nitrobacter* along with the usual flora and fauna which balance this ecosystem. Denitrification may then occur to release atmospheric nitrogen or the nitrate ion, released by *Nitrobacter,* may be taken up by plants or some species of anaerobic bacteria where it is reduced to ammonium ion and incorporated into amino acids and other nitrogen–carbon containing compounds. To complete the cycle, atmospheric nitrogen is then fixed by nitrifying bacteria, either free living in the soil or in close harmony with plants as described earlier in this chapter.

Closing Remarks

The underpinning biochemistry and natural cycles described in this chapter form the basis of all environmental biotechnological interventions, and a thorough appreciation of them is an essential part of understanding the practical applications which make up most of the rest of this work.

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Case Study 2.1 Environmental Health Laboratory (Middlesborough, England)

Some forms of environmental contamination are not, by their very nature, immediately apparent. This is particularly true when it is of microbial origin. The Environmental Health Laboratory of the Public Health Laboratory Service (PHLS) at The James Cook University Hospital, Middlesborough, is responsible for environmental microbial analysis. Materials most commonly examined are from water originating from the sea, rivers, spa pools, swimming pools and drinking water. They also analyse food in cases of suspected food poisoning and swabs from a variety of possibly contaminated surfaces. On receipt of samples at the PHLS, they are recorded with full details of time and place of sampling and are given identification codes. The laboratory insists that the specimens are transported in such a manner as to prevent bacterial multiplication. For some bacteria a resuscitation step is included to aid the recovery of damaged organisms. This can be important to ensure that all microbes present in the original material remain viable and culturable at least until testing has been accomplished. The total concentration of bacteria is determined by transferring a measured volume of the sample onto a petri dish containing growth medium solidified in agar. After these 'plates' have been incubated, usually overnight, colonies are visible with the naked eye, each one originating from a single bacterium. These colonies are counted and the number of organisms in each millilitre of original sample, calculated. There are many specific tests which can be used to identify the bacteria. The decision of which to use routinely, has been determined partly by constraints of time since a speedy warning is essential if pathogens or a particularly high microbial load is present. In addition, the tests are designed to look for the microbes which are specially problematic in given circumstances such as Pseudomonads in swimming and spa pools, or which are indicative of a particular contamination. An example of the latter are faecal coliforms, such as *Escherichia coli*, which can indicate the presence of sewage. A rapid test for these involves incubation of a measured volume of sample with the colourless chemical orthonitrophenol galactoside (ONPG). If the enzyme *β-galactosidase*, expressed by these coliforms, is present, galactose is hydrolysed from ONPG which produces a yellow colour. This is one of many rapid tests which rely on colour change in response to the activity of an enzyme diagnostic of a group of organisms, and provides for a simple and reproducible method of scoring for the presence or absence of contamination. If these initial tests prove positive within the guidelines set for

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public health, further detailed analyses are performed as necessary to determine the extent and identity of the contamination.

Inevitably, much of routine testing seems rather dull, but this essential service offered by the PHLS is vital for an early warning of system malfunction, hopefully preventing a minor problem becoming a major incident.

3

Fundamentals of Biological Intervention

The manipulation of natural cycles lies at the heart of much environmental biotechnology and engineering solutions to the kinds of problem for which this technology is appropriate, typically centres on adapting existing organisms and their inherent abilities. For the most part, the sorts of 'environmental' problems that mankind principally concerns itself about, are those which exist in the portion of the biosphere which most directly affects humanity itself. As a result, most of the organisms used share many of our own needs and the majority of the relevant cycles are ones which are, at least, largely familiar. While other aspects of biotechnology may demand techniques of molecular biology and genetic manipulation, as has been discussed, the applications of biological science, certainly to questions of pollution and waste, generally do not. Their position in respect of the third leg of the intervention tripod, shown in Figure 1.1, in clean manufacturing, is more ambiguous and there is distinct scope for them to have a greater role here, in the future. However, while this undoubtedly represents a contribution in terms of reduced pollution or the minimisation of waste, with regard to the express demands for environmental amelioration, their involvement is, at best, marginal. This is not to say that genetically manipulated organisms (GMOs) have no relevance to the field, but rather that, on the whole, they are greatly eclipsed in much of current practice by rather more ordinary organisms.

Using Biological Systems

Consequently, a number of themes and similarities of approach exist, which run as common and repeated threads throughout the whole of the science. Thus, optimisation of the activities of particular organisms, or even whole biological communities, to bring about any desired given end, typically requires manipulation of local conditions. Control of temperature, the accessibility of nutrients and the availability of oxygen are commonly the tools employed, especially when the target effectors are microbes or isolated biological derivatives. For the kind of whole organism approaches typified by phytotechnological interventions discussed in Chapter 7, this may prove a more difficult proposition, but nevertheless, one which still remains relevant at least in principle. The typical factors affecting

the use of biological systems in environmental engineering relate to the nature of the substances needing to be removed or treated and to the localised environmental conditions pertaining to the particular situation itself. Thus, in respect of the former, the intended target of the bioprocessing must generally be both susceptible and available to biological attack, in aqueous solution, or at least in contact with water, and within a low to medium toxicity range. Generally, the local environmental conditions required would ideally offer a temperature of 20–30 °C but a range of 0–50 °C will be tolerated in most cases, while an optimum pH lies in the range 6.5–7.5, but again a wider tolerance of 5.0–9.0 may be acceptable, dependent on the precise organism involved. For land-based applications, especially in the remediation of contamination or as a component of integrated pollution control measures, there is an additional common constraint on the substrate. Typically the soil types best suited to biotechnological interventions are sands and gravels, with their characteristically low nutrient status, good drainage, permeability and aeration. By contrast, biological treatments are not best suited to use in clays or peat or other soils of high organic content. In addition, generalised nutrient availability, oxygenation and the presence of other contaminants can all play a role in determining the suitability of biological intervention for any given application.

Extremophiles

As has been previously mentioned, in general the use of biotechnology for environmental management relies on mesophilic micro-organisms which have roughly similar environmental requirements to ourselves, in terms of temperature, pressure, water requirement and relative oxygenation. However, often some of their abilities, which are directly instrumental in enabling their use in this context, arose in the first instance as a result of previous environmental pressures in the species (pre)history. Accordingly, ancient metabolic pathways can be very valuable tools for environmental biotechnology. Thus, the selective advantages honed in Carboniferous coal measures and the Pleistocene tar pits have produced microbes which can treat spilled mineral oil products in the present and methanogenesis, a process developed by the Archae during the dawn of life on earth, remains relevant to currently commonplace biological interventions. Moreover, some species living today tolerate extreme environments, like high salinity, pressures and temperatures, which might be of use for biotech applications requiring tolerance to these conditions. The Archaea (the group formerly known as the archaebacteria and now recognised as forming a distinct evolutionary line) rank amongst their numbers extreme thermophiles and extreme halophiles in addition to the methanogens previously mentioned. Other species tolerate high levels of ionising radiation, pH or high pressures as found in the deep ocean volcanic vents known as ‘black smokers’.

Making use of these extremophile organisms could provide a way of developing alternative routes to many conventional chemicals or materials in such a way

as to offer significant advantages over existing traditional processes. Many current industrial procedures generate pollution in one form or another and the challenge of such 'green chemistry' is to design production systems which avoid the potential for environmental contamination. The implementation of 'clean manufacturing technologies' demands considerable understanding, innovation and effort if biologically derived process engineering of this kind is to be made a reality. With environmental concerns placing ever growing emphasis on energy efficiency and low carbon usage, industrial applications of the life sciences in this way seem likely to be increasingly relevant. To date, however, there has been little commercial interest in the extremophiles, despite their very obvious potential for exploitation.

The existence of microbes capable of surviving in extreme environments has been known since the 1960s, but the hunt for them has taken on added impetus in recent years as possible industrial applications for their unique biological capabilities have been recognised. As might be expected, much of the interest centres on the extremophile enzymes, the so-called 'extremozymes', which enable these species to function in their demanding natural habitats. The global market for enzymes amounts to around $3 billion (US) annually for biomedical and other industrial uses and yet the 'standard' enzymes typically employed cease working when exposed to heat or other extreme conditions. This often forces manufacturing processes that rely on them to introduce special steps to protect the proteins during either the active stage or storage. The promise of extremozymes lies in their ability to remain functional when other enzymes cannot. The potential for the mass use of enzymatic 'clean production' is discussed more fully in the following chapter, but the major benefit of using extremophile enzymes in this role is that they offer a way to obviate the requirement for such additional procedures, which inevitably both increases process efficiency and reduces costs. In addition, their novel and distinct abilities in challenging environments allows them to be considered for use as the basis of entirely new enzyme-based approaches to processing. Such methods, if properly designed and implemented, have the potential to give rise to major environmental and economic benefits compared with traditional energy-intensive chemical procedures. However, the widespread uptake and integration of biocatalytic systems as industrial production processes in their own right is not without obstacles which need to be overcome. In many conventional catalytic processes, chemical engineers are free to manipulate turbulence, pH, temperature and pressure for process intensification, often using a variety of reactor configurations and regimes to bring about the desired enhancement of productivity (Wright and Raper 1996). By contrast, in biological systems, the use of turbulence and other such conventional intensification methods is not appropriate as the microbial cells are typically too sensitive to be subjected to this treatment, as are the isolated enzymes. Such procedures often irreversibly denature proteins, destroying enzymatic activity.

Thermophiles

Of all the extremophiles, thermophiles are amongst the best studied, thriving in temperatures above 45 °C, while some of their number, termed hyperthermophiles, prefer temperatures in excess of 85 °C. Unsurprisingly, the majority of them have been isolated from environments which have some association with volcanic activity. The first extremophile capable of growth at temperatures greater than 70 °C was identified in the late 1960s as a result of a long-term study of life in the hot-springs of Yellowstone National Park, Wyoming, USA, headed by Thomas Brock of the University of Wisconsin-Madison. Now known as *Thermus aquaticus*, this bacterium would later make possible the widespread use of a revolutionary technology, the polymerase chain reaction (PCR), which is returned to later in this chapter. Shortly after this initial discovery, the first true hyperthermophile was found, this time an archaean which was subsequently named *Sulfolobus acidocaldarius*. Having been discovered in a hot acidic spring, this microbe thrives in temperatures up to 85 °C. Hyperthermophiles have since been discovered from deep sea vent systems and related features such as geothermal fluids, attached sulphide structures and hot sediments. Around 50 species are presently known. Some grow and reproduce in conditions hotter than 100 °C, the current record being held by *Pyrolobus fumarii*, which was found growing in oceanic 'smokers'. Its optimum temperature for reproduction is around 105 °C but will continue to multiply up to 113 °C. It has been suggested that this represents merely the maximum currently accepted for an isolated and culturable hyperthermophile and is probably not even close to the upper temperature limit for life which has been postulated at around 150 °C, based on current understanding. Although no one knows for certain at this time, it is widely thought that, higher than this, the chemical integrity of essential molecules will be unlikely to escape being compromised.

To set this in context, isolated samples of commonplace proteins, like egg albumin, are irreversibly denatured well below 100 °C. The more familiar mesophilic bacteria enjoy optimum growth between 25–40 °C; no known multicellular organism can tolerate temperatures in excess of 50 °C and no eukaryotic microbe known can survive long-term exposure to temperatures greater than around 60 °C. The potential for the industrial exploitation of the biochemical survival mechanisms which enable thermo- and hyperthermophiles to thrive under such hot conditions is clear. In this respect, the inactivation of thermophiles at temperatures which are still too hot for other organisms to tolerate may also have advantages in commercial processes. Though an extreme example in a world of extremes, the previously mentioned *P. fumarii*, stops growing below 90 °C; for many other species the cut-off comes at around 60 °C.

A good understanding of the way in which extremophile molecules are able to function under these conditions is essential for any future attempt at harnessing the extremozymes for industrial purposes. One area of interest in particular is how the structure of molecules in these organisms, which often very closely resemble

their counterparts in mesophilic microbes, influences activity. In a number of heat-tolerant extremozymes, for example, the major difference appears to be no more than an increased prevalence of ionic bonds within the molecule.

Though the industrial use of extremophiles in general has been limited to date, it has notably given rise to polymerase chain reaction (PCR), a major technique used in virtually every molecular biology laboratory worldwide. The application of PCR has, in addition, opened the flood gates for the application of genetic analyses in many other branches of life science, including forensics and medical diagnosis. Though this is a tool of genetic engineering rather than anything which could be argued as an 'environmental' application, it does illustrate the enormous potential of extremozymes. The process uses a DNA polymerase, called Taq polymerase, derived from *T. aquaticus*, as mentioned earlier, and was invented by Kary Mullins in the mid-1980s. The original approach relied on mesophilic polymerases and since the reaction mixture is alternately cycled between low and high temperatures, enzymatic denaturation took place, requiring their replenishment at the end of each hot phase. Samples of *T. aquaticus* had been deposited shortly after the organism's discovery, some 20 years earlier, and the isolation of its highly heat-tolerant polymerase enabled totally automated PCR technology to be developed. In recent years, some PCR users have begun to substitute Pfu polymerase, isolated from another hyperthermophile, *Pyrococcus furiosus*, which has an optimum temperature of 100 °C.

Other extremophiles

As was stated earlier, the thermophiles are amongst the best investigated of the extremophiles, but there are many other species which survive under equally challenging environmental conditions and which may also have some potential as the starting point for future methods of reduced pollution manufacturing. For example, cold environments are more common on earth than hot ones. The average oceanic temperature is around 1–3 °C and vast areas of the global land mass are permanently or near-permanently frozen. In these seemingly inhospitable conditions, extremophiles, known as psychrophiles, flourish. A variety of organisms including a number of bacteria and photosynthetic eukaryotes can tolerate these circumstances, often with an optimum functional temperature as low as 4 °C and stopping reproduction above 12 or 15 °C. Intensely saline environments, such as exist in natural salt lakes or within the artificial confines of constructed salt evaporation ponds are home to a group of extremophiles, termed the halophiles. Under normal circumstances, water flows from areas of low solute concentration to areas where it is higher. Accordingly, in salty conditions, unprotected cells rapidly lose water from their cytoplasm and dehydrate. Halophilic microbes appear to deal with this problem by ensuring that their cytoplasm contains a higher solute concentration than is present in their surroundings. They seem to achieve this by two distinct mechanisms, either manufacturing large quantities of solutes for themselves or concentrating a solute extracted from external sources.

A number of species, for example, accumulate potassium chloride (KCl) in their cytoplasm, with the concomitant result that extremozymes isolated from these organisms will only function properly in the presence of high KCl levels. By the same token, many surface structural proteins in halophiles require severely elevated concentrations of sodium salts.

Acidophiles thrive in the conditions of low pH, typically below 5, which occur naturally as a result of sulphurous gas production in hydrothermal vents and may also exist in residual spoils from coal-mining activity. Though they can tolerate an externally low pH, an acidic intra-cellular environment is intolerable to acidophilic organisms, which rely on protective molecules in, or on, their cell walls, membranes or outer cell coatings to exclude acids. Extremozymes capable of functioning below pH1 have been isolated from these structures in some acidophile species.

At the other end of the spectrum, alkaliphiles are naturally occurring species which flourish in soda lakes and heavily alkaline soils, typically enduring pH9 or more. Like the previous acidophiles, alkaliphiles require more typically neutral internal conditions, again relying on protective chemicals on or near their surfaces or in their secretions to ensure the external environment is held at bay.

Diverse degradative abilities

Bacteria possessing pathways involved in the degradation of a number of organic molecules of industrial importance, have been acknowledged for some time. One oft-quoted example is that for toluene degradation in *Pseudomonas putida*, which exhibits a fascinating interplay between the genes carried on the chromosome and the plasmids (Burlage, Hooper and Sayler 1989). Bacteria are constantly being discovered which exhibit pathways involved in the degradation and synthesis of chemicals of particular interest to environmental biotechnologists. For example, a new class of biopolymer produced by the bacterium, *Ralstonia eutropha*, containing sulphur in its backbone, has recently been identified. (Lütke-Eversloh *et al*. 2001) It is possible that these and other novel biopolymers awaiting discovery, will have innovative and exciting applications in clean technology.

In very recent years, bacteria representing very diverse degradative abilities have been discovered in a variety of niches adding almost daily, to the pool of organisms of potential use to environmental biotechnology. By illustration these include phenol-degrading *Oceanomas baumannii* isolated from estuarine mud from the mouth of the River Wear, UK (Brown, Sutcliffe and Cummings 2001), chloromethane utilising *Hyphomicrobium* and *Methylobacterium* from polluted soil near a petrochemical factory in Russia (McDonald *et al*. 2001) and a strain of *Clostridium* able to degrade cellulose, isolated from soil under wood chips or the forest floor in northeast USA. In addition to their cellulytic activity, these *Clostridia* were also found to be mesophilic, nitrogen-fixing, spore-forming and obligate anaerobes (Monserrate, Leschine and Canale-Parola 2001). Again, there is interest in this organism with regard to clean technology in the hope that it may

be used to convert cellulose into industrially useful substances. A note of caution is that cellulose is a major product of photosynthesis and, being the most abundant biopolymer on this planet, has a vital role to play in the carbon cycle. Large-scale disturbance of this balance may have consequences to the environment even less welcome than the technologies they seek to replace. However, judicious use of this biotechnology could reap rewards at many levels.

Bacteria have also adapted to degrade man-made organics called xenobiotics.

Xenobiotics and Other Problematic Chemicals

The word is derived from the Greek '*xenos*' meaning foreign. Throughout this book the definition used is that xenobiotics are compounds which are not produced by a biological procedure and for which no equivalent exists in nature. They present a particular hazard if they are subject to bioaccumulation especially so if they are fat soluble since that enables them to be stored in the body fat of organisms providing an obvious route into the food chain. Despite the fact that these chemicals are man made, they may still be degraded by micro-organisms if they fit into one of the following regimes; gratuitous degradation, a process whereby the xenobiot resembles a natural compound sufficiently closely that it is recognised by the organism's enzymes and may be used as a food source, or cometabolism where the xenobiot is degraded again by virtue of being recognised by the organism's enzymes but in this case its catabolism does not provide energy and so cannot be the sole carbon source. Consequently, cometabolism may be sustained only if a carbon source is supplied to the organism. The ability of a single compound to be degraded can be affected by the presence of other contaminants. For example, heavy metals can affect the ability of organisms to grow, the most susceptible being Gram positive bacteria, then Gram negative. Fungi are the most resistant and actinomycetes are somewhere in the middle. This being the case, model studies predicting the rate of contaminant degradation may be skewed in the field where the composition of the contamination may invalidate the study in that application. Soil micro-organisms in particular are very versatile and may quickly adapt to a new food source by virtue of the transmission of catabolic plasmids. Of all soil bacteria, Pseudomonads seem to have the most highly developed ability to adapt quickly to new carbon sources. In bacteria, the genes coding for degradative enzymes are often arranged in clusters, or operons, which usually are carried on a plasmid. This leads to very fast transfer from one bacterium to another especially in the case of *Pseudomonas* where many of the plasmids are self-transmissible. The speed of adaptation is due in part to the exchange of plasmids but in the case of the archaeans particularly, the pathways they carry, which may have been latent over thousands of bacterial generations, owe their existence to previous exposure over millions of years to an accumulated vast range of organic molecules. It is suggested that, unless there has been evolutionary pressure to the contrary, these latent pathways are retained to

a large extent requiring little modification if any to utilise new xenobiotics. Even so, bioremediation may require that organisms are altered in some way to make them more suitable for the task and this topic is addressed in Chapter 9. Briefly, the pathways may be expanded by adaptation to the new molecule, or very much less commonly, wholescale insertion of 'foreign' genes may occur by genetic manipulation. There have been several cases reported where catabolic pathways have been expanded in the laboratory. Hedlund and Staley (2001) isolated a strain of *Vibrio cyclotrophicus* from marine sediments contaminated with creosote. By supplying the bacteria with only phenanthrene as a carbon and energy source, the bacteria were trained to degrade several PAHs although some of these only by cometabolism with a supplied carbon source.

Endocrine disrupters

To date, there are chemicals, including xenobiotics, which still resist degradation in the environment. This may be due to a dearth, at the site of contamination, of organisms able to degrade them fully or worse, microbial activity which changes them in such a way that they pose a bigger problem than they did previously. One such example is taken from synthetic oestrogens such as 17α-ethinyloestradiol commonly forming the active ingredient of the birth control pills, and the natural oestrogens which, of course, are not xenobiotics. Natural oestrogens are deactivated in humans by glucuronidation, as shown in Figure 3.1, which is a conjugation of the hormone with UDP-glucuronate making the compound more

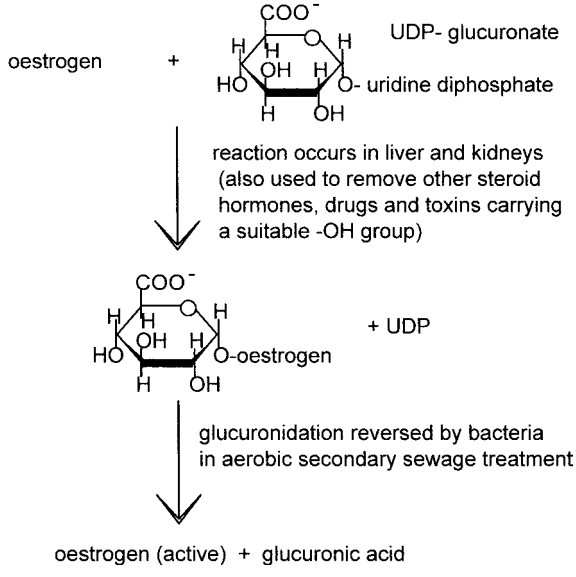

Figure 3.1 *Glucuronidation*

polar and easily cleared from the blood by the kidneys. It is in this modified and inactive form that it is excreted into the sewage. However, bacteria present in the aerobic secondary treatment in sewage treatment plants, have the enzyme, *β-glucuronidase*, which removes this modification thus reactivating the hormone.

As an aside, glucuronidation is not confined to hormones but is a process used to detoxify a number of drugs, toxins and carcinogens in the liver. The enzyme catalysing this process is induced in response to prolonged exposure to the toxin thus imparting increased tolerance or even resistance to the chemical.

Returning to the problem of elevated levels of active hormones in the waterways, another aspect is that steroids do not occur in bacteria, although they are present in fungi, and so bacteria lack the necessary pathways to allow complete degradation of these hormones at a rate compatible with the dwell time in sewage treatment plants. The consequence has been raised levels of reactivated oestrogen and 17α-ethinyloestradiol in the waterways leading to disturbances of the endocrine, or hormonal, system in fauna downstream from sewage treatment plants. Such disturbances have been monitored by measuring the presence of the protein vitellogenin (Sole *et al*. 2001) which is a precursor to egg yolk protein, the results of which have indicated feminisation of male fish in many species including minnows, trout and flounders. The source of environmental oestrogens is not confined to outfall from sewage treatment plants, however, the fate of endocrine disrupters, examples of which are given in Figure 3.2, in sewage treatment plants is the subject of much research (Byrns 2001). Many other chemicals, including polyaromatic hydrocarbons (PAHs), dichlorodiphenyltrichloroethane (DDT), alkyl phenols and some detergents may also mimic the activity of oestrogen. There is general concern as to the ability of some organisms to accumulate these endocrine disrupters in addition to the alarm being raised as to the accumulative effects on humans of oestrogen-like activity from a number of xenobiotic sources.

To date there is no absolute evidence of risk to human health but the Environmental Agency and Water UK are recommending the monitoring of environmental oestrogens in sewage treatment outfall. Assays are being developed further to make these assessments (Gutendorf and Westendorf 2001) and to predict potential endocrine disrupter activity of suspected compounds (Takeyoshi *et al* 2002). Oestrogen and progesterone are both heat labile. In addition, oestrogen appears to be susceptible to treatment with ultra-violet light, the effects of which are augmented by titanium dioxide (Eggins 1999). The oestrogen is degraded completely to carbon dioxide and water thus presenting a plausible method for water polishing prior to consumption.

Another method for the removal of oestrogens from water, in this case involving *Aspergillus*, has also been proposed (Ridgeway and Wiseman 1998). Sulphation of the molecule by isolated mammalian enzymes, as a means of hormone inactivation is also being investigated (Suiko 2000). Taken overall, it seems unlikely that elevated levels of oestrogen in the waterways will pose a problem

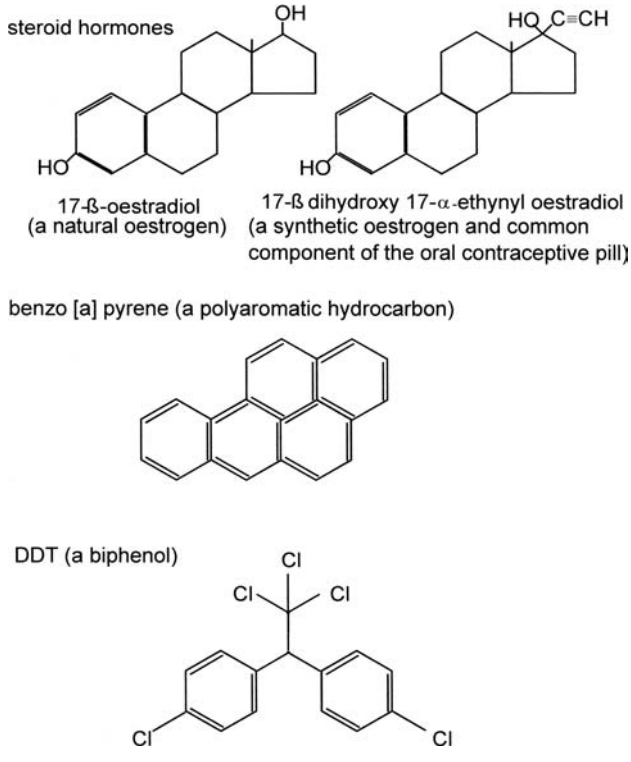

Figure 3.2 *Endocrine disrupters*

to human health in drinking water although, this does not address the problem affecting hormone-susceptible organisms living in contaminated water and thus exposed to this potential hazard.

New discoveries

Almost daily, there are novel bacteria being reported in the literature which have been shown to have the capacity to degrade certain xenobiots. Presumably the mutation which occurred during the evolution of the organism conferred an advantage, and selective pressure maintained that mutation in the DNA, thus producing a novel strain with an altered phenotype. Some example of such isolates are described here. Reference has already been made to some PAHs mimicking oestrogen which earns those chemicals the title of 'endocrine disrupters'. This is in addition to some being toxic for other reasons and some being carcinogenic or teratogenic. The PAHs are derived primarily from the petrochemicals industry and are polycyclic hydrocarbons of three or more rings which include as members, naphthalene and phenanthrene and historically have been associated

with offshore drilling, along with alkylphenols. Several genera of bacteria are now known to be able to degrade PAHs and recently, a novel strain of *Vibrio cyclotrophicus* able to digest naphthalene and phenanthrene, was isolated from creosote-contaminated marine sediments from Eagle Harbour, Washington, USA. It would appear that bacteria isolated from the same marine or estuarine environments may vary quite considerably in their abilities to degrade certain PAHs. This observation is viewed as indicative of diverse catabolic pathways demonstrated by these organisms and awaiting our full understanding (Hedlund and Staley 2001).

Polycyclic hydrocarbons (PCBs) are xenobiotics which, due to their high level of halogenation, are substrates for very few pathways normally occurring in nature. However, a strain of *Pseudomonas putida* able to degrade PCBs, was isolated recently from wastewater outflow from a refinery. This was achieved by the bacterium employing two pathways encoded by two separate operons; the *tod* pathway employed in toluene degradation, and the *cmt* pathway which normally is responsible for the catabolism of *p*-cumate which is a substituted toluene. The mutation which allowed this strain to utilise the *cmt* pathway was found to be a single base change to the promoter-operator sequence. This allowed all the enzymes in this pathway to be expressed under conditions where their synthesis would normally be repressed. Thus, the two pathways could work in conjunction with each other to metabolise PCBs, a relationship described as mosaic (Ohta *et al* 2001).

The pthalates are substituted single-ring phenols and include terephthalic acid and its isomers, the major chemicals used in manufacture of polyester fibres, films, adhesives, coatings and plastic bottles. In Chapter 2, and earlier in this chapter, homage has been paid to the resources of genetic capability exhibited by the archaeans. In a recent analysis of anaerobic sewage sludge, a methanogenic consortium of over 100 bacterial clones were found to have the capability to digest terephthalate. Characterisation of these by analysis of their ribosomal DNA sequences, revealed that almost 70% were archaeans most of which had not been previously identified, and that nearly 90% of the total bacteria comprised two of the novel archaean species. These two species are believed to be responsible for the degradation of terephthalic acid (Wu *et al* 2001). During wastewater treatment, terepthalic acid is usually treated by aerobic processes. However, this consortium, or others like it provide an anaerobic alternative which, being methanogenic, may be structured to offset processing costs by the utilisation of the methane.

Mobility of DNA

Throughout this book, reference is made to the movement of genes within and between organisms. The reason why it appears at all in a book on environmental biotechnology is to emphasise the ‘oneness’ of the environment, not just at the more obvious level of industrial impact but right down to the interaction

between the genetic material of organisms themselves. Plasmids may be transferred between bacteria by conjugation, of which there are several types, but all of which require direct cell to cell contact. Not only are genes transferred between bacteria on plasmids, but bacteriophages (bacterial viruses) are also vectors for intercellular transmission. Similarly, eukaryotic viruses are able to transfer genetic material between susceptible cells. In addition, bacterial cells may pick up DNA free in the environment under conditions where their cell wall has become 'leaky' to fragments of this macromolecule, a process called transformation. There is also considerable rearrangement of genomic material within an organism stimulated by the presence of transposons. There are many classes of transposable elements which are short pieces of DNA, able to excise themselves, or be excised, out of a genome. Often they take with them neighbouring pieces of DNA, and then reinsert themselves, sometimes with the assistance of other genes, into a second site distinct from the original location on the same genome. Insertion may be into specific sites or random, depending on the nature of the transposon. Transposition normally requires replication of the original DNA fragment and so a copy of this transposon is transferred leaving the original behind. Transposition is widespread and occurs in virtually all organisms for which evidence of this process has been sought, both prokaryotic and eukaryotic. The term 'transposable element', was first coined by Barbara MacClintock, who discovered them in maize, publishing her data in the early 1950s. However, it was not until many years later that the full significance of her work was being recognised, with similar elements being discovered in bacteria. Transposable elements are known to promote the fusion of plasmids within a bacterial cell, where more than one type of plasmid is present. The fusion is stimulated by the presence of insertion sequences (IS), which are short pieces of DNA of a defined and limited range of sequences. They are often found at either end of a transposable element. Their presence enables various DNA rearrangements to take place leading to moderation of gene expression. Taking together the reorganisation of DNA within all types of organisms attributable to transposable elements and IS, with transfer of DNA between organisms by plasmids and transformation, in the case of prokaryotes, and viruses in the case of both prokaryotes and eukaryotes, the potential for DNA rearrangement within and between organisms is enormous.

It has been proposed (Reanney 1976), that such transfer is far more universal than had previously been voiced. Transfer of genes by extra chromosomal elements (ECEs), which is the all-embracing name given to include plasmids and viruses, models the means by which molecular evolution takes place in the environment. The proposal is that the evolutionary process occurs principally by insertions and deletions of the genome such as those caused by the activities of ECEs and transposable elements and not by point mutations more frequently observed in isolated cultures such as those maintained in laboratory conditions. It is further suggested that much of the phenotypic novelty seen in evolution is the result of rearrangement of existing structural genes into a different region

of the genome and therefore operating under different parameters affecting gene regulation. Transfer of genes across wide taxonomic gaps is made possible by the mobile nature of ECEs many of which may cross species barriers often resulting in the insertion of all or part of the ECE into the recipient genome. Examples of such mobility are viruses which infect a wide host range, such as some retroviruses, the alfalfa mosaic virus, and the Ti plasmid of *Agrobacterium tumefaciens* which the bacterium introduces into plant cells. The retroviruses, of which Human Immunodeficiency Virus (HIV) is an example, are unusual in having RNA as their genetic material. They replicate in a manner which includes double-stranded DNA as an intermediate and so may integrate into the host cell genome. RNA viruses tend to be more susceptible than DNA viruses to mutation presumably due to the less chemically stable nature of the macromolecule. They have been invoked by Reanney (1976) as being the likely agents for the spread of genetic information between unrelated eukaryotes. His observations led him to conclude that there is only a blurred distinction between cellular and ECE DNA both in eukaryotes and prokaryotes and further suggest that no organism lives in true genetic isolation as long as it is susceptible to at least one of the classes of ECEs described above. Clearly, for the mutation to be stabilised, it must occur in inheritable DNA sequences, a situation reasonably easy to achieve in microbes and at least possible in multicellular organisms.

The existence of genetic mobility has been accepted for many years, even though the extent and the mechanisms by which it operates are still being elucidated. From this knowledge several lessons may be learned; among them, that the genetic environment of any organism may well be significant and that there is some justification in viewing the principle of genetic engineering as performing in the laboratory, a process which is occurring in abundance throughout the living world. This topic is explored further in Chapters 9, 10 and 11.

Closing Remarks

As has been seen, even within the brief discussion in this chapter, life on Earth is a richly varied resource and the functional reality of biodiversity is that many more metabolic pathways exist, particularly within the microbial melting pot, than might be commonly supposed. As a result, a number of generally unfamiliar groups of chemicals and organisms have implications for the application of environmental biotechnology which exceed their most obvious contributions to a wider consideration of the life sciences. Hence, xenobiotics, as an example of the former, represent a current problem for which the solution remains unresolved and extremophiles, as the latter, hold the potential to revolutionise many industrial procedures, thereby heralding major benefits in terms of ‘clean technology’. There are many aspects of current environmental management for which there is no presently relevant biotechnological intervention. However, this is not a static science, either in terms of what can be done, or the tools available. Discoveries

and developments both within the field and from other disciplines can and do filter in and alter the state of the possible. Thus, while the bulk of the rest of this book addresses the sorts of biotechnological applications routinely applied to deal with today's environmental problems, it must be borne in mind that some of the issues described in this section may well assume greater relevance in the future and this is a point which will, at least in part, be reviewed in the final chapter.

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Case Study 3.1 Feminisation of Fish (England and Wales)

Of all the endocrine disrupters, perhaps most concern has been voiced over the possible consequences of synthetic oestrogens. The most recent study into hormone disruption of fish in the UK, published by the Environment Agency (EA), has revealed that oestrogenic steroids in sewage effluent are more potent in this respect than had been previously thought. As result of this five-year investigation of 10 river catchments in England and Wales which receive sewage treatment effluent, it has become clear that the reproductive health of male fish is being adversely affected. With nearly half of all those studied either having eggs in their testes or developed female reproductive ducts, 25% producing damaged sperm, and 10% being sterile, the future sustainability of fish populations in a number of rivers in England and Wales could be at risk.

Many oestrogens, both natural and manufactured, have been implicated, but the synthetic steroid 17α ethinyloestradiol, which is present in the urine of women using the contraceptive pill, is of particular concern, producing effects in fish at concentrations below 1 μg/l.

While the resulting level of concern over this 'feminisation' is likely to drive the development of a risk management strategy for oestrogens in sewage effluent, which may ultimately necessitate changes in treatment practice or technology, it is clear that not all fish populations are exposed to the same degree and so careful targeting of any intended action will be essential.

The research, which was undertaken by scientists from Brunel and Exeter Universites, and funded by the EA, the Department for Environment, Food and Rural Affairs (DEFRA) and the Natural Environment Research Council (NERC), focused on wild populations of roach (*Rutilus rutilus*) and gudgeon (*Gobio gobio*). It specifically examined the causes and consequences of feminisation in these species and, while it is expected that the situation is similar for other fish species, it was not designed to draw conclusions regarding likely human health effects. In the UK, around a third of

Continued on page 64

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drinking water is abstracted from rivers and, in common with many other industrialised countries, human sperm counts are progressively dropping. The possibility of a connection between these and the clear evidence of endocrine disruption arising from the fish study has been raised in the media, but for the present at least, nothing has been established for certain.

However, such public health concerns aside, the persistence and potency of synthetic oestrogens remains a clear example of the environmental importance of xenobiotic contamination.

4

Pollution and Pollution Control

Pollution has become one of the most frequently talked about of all environmental problems by the world at large and yet, in many respects, it can often remain one of the least understood. The word itself has a familiar ring to it and inevitably the concept of pollution has entered the wider consciousness as a significant part of the burgeoning 'greening' of society in general. However, the diverse nature of potentially polluting substances can lead to some confusion. It is important to realise that not all pollutants are manufactured or synthetic, that under certain circumstances, many substances may contribute to pollution and that, perhaps most importantly for our purposes, any biologically active substance has the potential to give rise to a pollution effect. This inevitably leads to some difficulty in any attempt at classifying pollutants, since clearly, they do not represent a single unified class, but rather a broad spectrum. While it is possible, as we shall discuss shortly, to produce a means of systematic characterisation of pollutant substances, though useful for a consideration of wider contamination effects, this is an inherently artificial exercise. It is, therefore, perhaps more useful to begin the discussion with a working definition.

The UK Environmental Protection Act (EPA) 1990 statutorily offers the following:

> 'Pollution of the environment' means pollution of the environment due to the release (into any environmental medium) from any process of substances which are capable of causing harm to man or any other living organisms supported by the environment.
>
> *EPA, Introduction*

> ....the escape of any substance capable of causing harm to man or any other living organism supported by the environment
>
> *EPA, Section 29, Part II*

In essence, then, pollution is the introduction of substances into the environment which, by virtue of their characteristics, persistence or the quantities involved, are likely to be damaging to the health of humans, other animals and plants, or otherwise compromise that environment's ability to sustain life. It should be obvious that this is an expressly inclusive definition, encompassing not simply the obviously toxic or noxious substances, but also other materials which can have a polluting effect under certain circumstances.

Classifying Pollution

While, as we said earlier, this diverse nature of potential pollutants makes their systematisation difficult in absolute terms, it is possible to produce functional classifications on the basis of various characteristics. However, it must be clearly borne in mind that all such classification is essentially artificial and subjective, and that the system to be adopted will typically depend on the purpose for which it is ultimately intended. Despite these limitations, there is considerable value in having some method, if only as a predictive environmental management tool, for considerations of likely pollutant effect.

Classification may, for example, be made on the basis of the chemical or physical nature of the substance, its source, the environmental pathway used, the target organism affected or simply its gross effect. Figure 4.1 shows one possible example of such a categorisation system and clearly many others are possible.

The consideration of a pollutant's properties is a particularly valuable approach when examining real-life pollution effects, since such an assessment requires both the evaluation of its general properties and the local environment. This may include factors such as:

- toxicity;
- persistence;
- mobility;
- ease of control;
- bioaccumulation;
- chemistry.

Toxicity

Toxicity represents the potential damage to life and can be both short and long term. It is related to the concentration of pollutant and the time of exposure to it, though this relationship is not an easy one. Intrinsically highly toxic substances can kill in a short time, while less toxic ones require a longer period of exposure to do damage. This much is fairly straightforward. However, some pollutants which

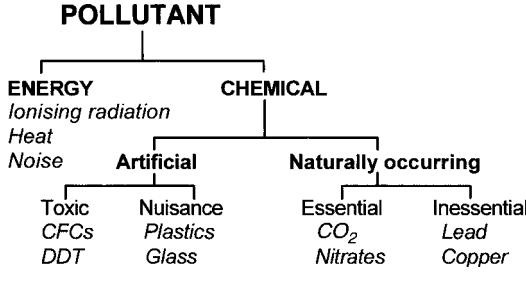

Figure 4.1 *Pollution classification*

may kill swiftly in high concentrations, may also have an effect on an organism's behaviour or its susceptibility to environmental stress over its lifetime, in the case of low concentration exposure.

Availability also features as an important influence, both in a gross, physical sense and also in terms of its biological availability to the individual organism, together with issues of its age and general state of health. Other considerations also play a significant part in the overall picture of toxicity and we shall return to look at some of them in greater depth shortly.

Persistence

This is the duration of effect. Environmental persistence is a particularly important factor in pollution and is often linked to mobility and bioaccumulation. Highly toxic chemicals which are environmentally unstable and break down rapidly are less harmful than persistent substances, even though these may be intrinsically less toxic.

Mobility

The tendency of a pollutant to disperse or dilute is a very important factor in its overall effect, since this affects concentration. Some pollutants are not readily mobile and tend to remain in 'hot-spots' near to their point of origin. Others spread readily and can cause widespread contamination, though often the distribution is not uniform. Whether the pollution is continuous or a single event, and if it arose from a single point or multiple sources, form important considerations.

Ease of control

Many factors contribute to the overall ease with which any given example of pollution can be controlled, including the mobility of the pollutant, the nature, extent or duration of the pollution event and local site-specific considerations. Clearly, control at source is the most effective method, since it removes the problem at its origin. However, this is not always possible and in such cases, containment may be the solution, though this can itself lead to the formation of highly concentrated hot-spots. For some substances, the dilute and disperse approach, which is discussed more fully later in this chapter, may be more appropriate, though the persistence of the polluting substances must obviously be taken into account when making this decision.

Bioaccumulation

As is widely appreciated, some pollutants, even when present in very small amounts within the environment, can be taken up by living organisms and become concentrated in their tissues over time. This tendency of some chemicals to be taken up and then concentrated by living organisms is a major consideration, since even relatively low background levels of contamination may accumulate up the food chain.

Chemistry

Pollution effects are not always entirely defined by the initial nature of the contamination, since the reaction or breakdown products of a given pollutant can sometimes be more dangerous than the original substance. This is of particular relevance to the present discussion, since the principle underlying much of practical bioremediation in general involves the break down of pollutants to form less harmful products.

This is further complicated in that while the chemistry of the pollutant itself is clearly important, other substances present and the geology of the site may also influence the outcome. Accordingly, both synergism and antagonism are possible. In the former, two or more substances occurring together produce a combined pollution outcome which is greater than simply the sum of their individual effects; in the latter, the combined pollution outcome is smaller than the sum of each acting alone.

The Pollution Environment

There is sometimes a tendency for contamination to be considered somewhat simplistically, in isolation from its context. It is important to remember that pollution cannot properly be assessed without a linked examination of the environment in which it occurs. The nature of the soil or water which harbours the pollution can have a major effect on the actual expressed end-result. In the case of soil particularly, many properties may form factors in the modification of the contamination effect. Hence, the depth of soil, its texture, type, porosity, humus content, moisture, microbial complement and biological activity can all have a bearing on the eventual pollution outcome. Inevitably, this can make accurate prediction difficult, though a consideration of system stability can often give a good indication of the most likely pollution state of a given environment.

The more stable and robust the environmental system affected, the less damage a given pollution event will inflict and clearly, fragile ecosystems or sensitive habitats are most at risk. It should be obvious that, in general terms, the post-pollution survival of a given environment depends on the maintenance of its natural cycles. Equally obviously, artificial substances which mimic biological molecules can often be major pollutants since they can modify or interrupt these processes and pollution conversion can spread or alter the effect.

Pollution Control Strategies

Dilution and dispersal

The concept of ‘dilute and disperse’ was briefly mentioned earlier in this discussion. In principle, it involves the attenuation of pollutants by permitting them to become physically spread out, thereby reducing their effective point concentration. The dispersal and the consequent dilution of a given substance

depends on its nature and the characteristics of the specific pathway used to achieve this. It may take place, with varying degrees of effectiveness, in air, water or soil.

Air

In general terms, air movement gives good dispersal and dilution of gaseous emissions. However, heavier particulates tend to fall out near the source and the mapping of pollution effects on the basis of substance weight/distance travelled is widely appreciated.

Water

Typically, there is good dispersal and dilution potential in large bodies of water or rivers, but smaller watercourses clearly have a correspondingly lower capacity. It is also obvious that moving bodies of water disperse pollutants more rapidly than still ones.

Soil

Movement through the soil represents another opportunity for the dilute and disperse approach, often with soil water playing a significant part, and typically aided by the activities of resident flora and fauna. The latter generally exerts an influence in this context which is independent of any bioaccumulation potential.

Concentration and containment

The principle behind this is diametrically opposed to the previous approach, in that instead of relying on the pollutant becoming attenuated and spread over a wide area, it is an attempt to gather together the offending substance and prevent its escape into the surrounding environment.

The inherent contradiction between these two general methods is an enduring feature of environmental biotechnology and, though the fashion changes from time to time, favouring first one and then the other, it is fair to say that there is a place for both, dependent on individual circumstances. As with so much relating to the practical applications of biotechnologies to environmental problems, the idea of a 'best' method, at least in absolute terms, is of little value. The whole issue is far more contextually sensitive and hence the specific modalities of the particular, are often more important concerns than the more theoretically applicable general considerations.

Practical Toxicity Issues

The general factors which influence toxicity have already been set out earlier in this discussion, but before moving on to consider wider practical issues it is helpful to mention briefly the manner in which the toxic action of pollutants arises.

There are two main mechanisms, often labelled 'direct' and 'indirect'. In the former, the effect arises by the contaminant combining with cellular constituents or enzymes and thus preventing their proper function. In the latter, the damage is done by secondary action resulting from their presence, typified by histamine reactions in allergic responses.

The significance of natural cycles to the practical applications of environmental biotechnology is a point that has already been made. In many respects the functional toxicity of a pollution event is often no more than the obverse aspect of this same coin, in that it is frequently an overburdening of existing innate systems which constitutes the problem. Thus the difficulty lies in an inability to deal with the contaminant by normal routes, rather than the simple presence of the substance itself. The case of metals is a good example. Under normal circumstances, processes of weathering, erosion and volcanic activity lead to their continuous release into the environment and corresponding natural mechanisms exist to remove them from circulation, at a broadly equivalent rate. However, human activities, particularly after the advent of industrialisation, have seriously disrupted these cycles in respect of certain metals, perhaps most notably cadmium, lead, mercury and silver. While the human contribution is, clearly, considerable, it is also important to be aware that there are additional potential avenues of pollution and that other metals, even though natural fluxes remain their dominant global source, may also give rise to severe localised contamination at times.

The toxicity of metals is related to their place in the periodic table, as shown in Table 4.1 and reflects their affinity for amino and sulphydryl groups (associated with active sites on enzymes).

In broad terms, type-A metals are less toxic than type-B, but this is only a generalisation and a number of other factors exert an influence in real-life situations. Passive uptake by plants is a two-stage process, beginning with an initial binding onto the cell wall followed by diffusion into the cell itself, along a concentration gradient. As a result, those cations which readily associate with particulates are accumulated more easily than those which do not. In addition, the presence of chelating ligands may affect the bio-availability and thus, the resultant toxicity of metals. Whereas some metal-organic complexes (Cu-EDTA for example) can detoxify certain metals, lipophilic organometallic complexes can increase uptake and thereby the functional toxic effect observed.

Table 4.1 *Metal periodicity and toxicity*

Metal group	Relative toxicity
Group IA	Na < K < Rb and Cs
Group IB	Cu < Ag < Au
Group IIA	Mg < Ca < Sr < Ba
Group IIB	Zn < Cd < Hg
Group IIIA	Al < Ga < In < Tl

Although we have been considering the issue of metal toxicity in relation to the contamination of land or water, it also has relevance elsewhere and may be of particular importance in other applications of biotechnologies to environmental problems. For example, anaerobic digestion is a engineered microbial process commonly employed in the water industry for sewage treatment and gaining acceptance as a method of biowaste management. The effects of metal cations within anaerobic bioreactors are summarised in Table 4.2, and from which it is apparent that concentration is the key factor.

However, the situation is not entirely clear cut as the interactions between cations under anaerobic conditions may lead to increased or decreased effective toxicity in line with the series of synergistic/antagonistic relationships shown in Table 4.3.

Toxicity is often dependent on the form in which the substance occurs and substances forming analogues which closely mimic the properties of essential chemicals are typically readily taken up and/or accumulated. Such chemicals are often particularly toxic as the example of selenium illustrates.

Often wrongly referred to as a toxic metal, and though it has some metallic properties, selenium is a nonmetal of the sulphur group. It is an essential trace element and naturally occurs in soils, though in excess it can be a systemic poison with the LD_{50} for certain selenium compounds being as low as 4 micrograms per kg body weight.

Table 4.2 *The effect of metal cations on anaerobic digestion*

Cation	Stimulatory	Moderately inhibitory	Strongly inhibitory
Sodium	100–200	3500–5500	8 000
Potassium	200–400	2500–4500	12 000
Calcium	100–200	2500–4500	8 000
Magnesium	75–150	1000–1500	3 000

Concentrations in mg/l

Table 4.3 *Effective toxicity and synergistic/antagonistic relationships*

Toxic cation	Synergistic	Antagonistic
Ammonium-N	Calcium Magnesium Potassium	Sodium
Calcium	Ammonium-N Potassium	Sodium Magnesium
Magnesium	Ammonium-N Calcium	Sodium Potassium
Potassium	–	Sodium
Sodium	Ammonium-N Calcium	Potassium

In plants, sulphur is actively taken up in the form of sulphate SO_4^{2-}. The similarity of selenium to sulphur leads to the existence of similar forms in nature, namely selenite, SeO_3^{2-} and selenate SeO_4^{2-}.

As a result, selenium can be taken up in place of sulphur and become incorporated in normally sulphur-containing metabolites.

Practical Applications to Pollution Control

In the next chapter contaminated land and bioremediation, which typically form a wider area of concern for environmental biotechnology, will be considered in some detail. To give a practical context with which to close this section, however, a brief discussion of air pollution and odour control follows.

Bacteria normally live in an aqueous environment which clearly presents a problem for air remediation. Frequently the resolution is to dissolve the contaminant in water, which is then subjected to bioremediation by bacteria, as in the following descriptions. However, there is scope for future development of a complementary solution utilising the fact that many species of yeast produce aerial hyphae which may be able to metabolise material directly from the air.

A variety of substances can be treated, including volatile organic carbon containing compounds (VOCs) like alcohols, ketones or aldehydes and odorous substances like ammonia and hydrogen sulphide (H_2S). While biotechnology is often thought of as something of a new science, the history of its application to air-borne contamination is relatively long. The removal of H_2S by biological means was first discussed as long ago as 1920 and the first patent for a truly biotech-based method of odour control was applied for in 1934. It was not until the 1960s that the real modern upsurge began, with the use of mineral soil filter media and the first true biofilters were developed in the succeeding decade. This technology, though refined, remains in current use. The latest state-of-the-art developments have seen the advent of the utilisation of mixed microbial cultures to degrade xenobiotics, including chlorinated hydrocarbons like dichloromethane and chlorobenzene.

A number of general features characterise the various approaches applied to air contamination. Typically systems run at an operational temperature within a range of 15–30 °C, in conditions of abundant moisture, at a pH between 6–9 and with high oxygen and nutrient availability. In addition, most of the substances which are commonly treated by these systems are water soluble.

The available technologies fall naturally into three main types, namely biofilters, biotrickling filters and bioscrubbers. To understand these approaches, it is probably most convenient to adopt a view of them as biological systems for the purification of waste or exhaust gases. All three can treat a wide range of flow rates, ranging from 1000–100 000 m^3/h, hence the selection of the most appropriate technology for a given situation is based on other criteria. The concentration of the contaminant, its solubility, the ease of process control and the land

requirement are, then, principal factors and they interact as shown in Table 4.4 to indicate the likely best approach.

Biofilters

As mentioned earlier, these were the first methods to be developed. The system, shown schematically in Figure 4.2, consists of a relatively large vessel or container, typically made of cast concrete, metal or durable plastic, which holds a filter medium of organic material such as peat, heather, bark chips and the like. The gas to be treated is forced, or drawn, through the filter, as shown in the diagram. The medium offers good water-holding capacity and soluble chemicals within the waste gas, or smelt, dissolve into the film of moisture around the matrix. Bacteria, and other micro-organisms present, degrade components of the resultant solution, thereby bringing about the desired effect. The medium itself provides physical support for microbial growth, with a large surface area to volume ratio, high in internal void spaces and rich in nutrients to stimulate and sustain bacterial activity. Biofilters need to be watered sufficiently to maintain optimum internal conditions, but waterlogging is to be avoided as this leads to compaction, and hence, reduced efficiency. Properly maintained, biofilters can reduce odour release by 95% or more.

Table 4.4 *Odour control technology selection table*

Technology	Compound concentration	Compound solubility	Process control	Land requirement
Biofilter	Low	Low	Low	High
Biotrickling filter	Low-medium	Low-high	Medium-high	Low
Bioscrubber	Low-medium	Medium-high	High	Low-medium

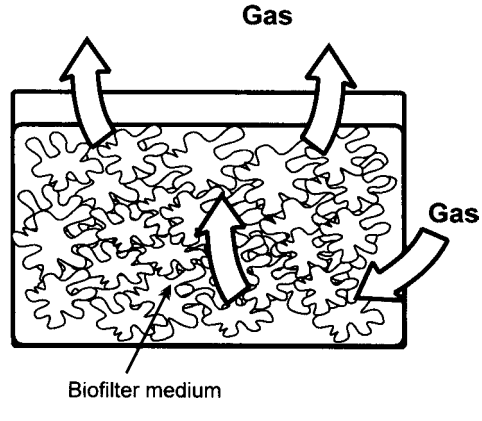

Figure 4.2 *Biofilter*

Biotrickling filters

As shown in Figure 4.3, in many respects these represent an intermediate technology between biofilters and bioscrubbers, sharing certain features of each. Once again, an engineered vessel holds a quantity of filter medium, but in this case, it is an inert material, often clinker or slag. Being highly resistant to compaction, this also provides a large number of void spaces between particles and a high surface area relative to the overall volume of the filter. The microbes form an attached growth biofilm on the surfaces of the medium. The odourous air is again forced through the filter, while water simultaneously recirculates through it, trickling down from the top, hence the name. Thus a counter-current flow is established between the rising gas and the falling water, as shown in the diagram, which improves the efficiency of dissolution. The biofilm communities feed on substances in the solution passing over them, biodegrading the constituents of the smell.

Process monitoring can be achieved relatively simply by directly sampling the water recirculating within the filter vessel. Process control is similarly straightforward, since appropriate additions to the circulating liquid can be made, as required, to ensure an optimum internal environment for bacterial action. Though the efficiency of the biotrickling filter is broadly similar to the previous method, it can deal with higher concentrations of contaminant and has a significantly smaller foot-print than a biofilter of the same throughput capacity. However, as with almost all aspects of environmental biotechnology, these advantages are obtained by means of additional engineering, the corollary of which is, inevitably, higher capital and running costs.

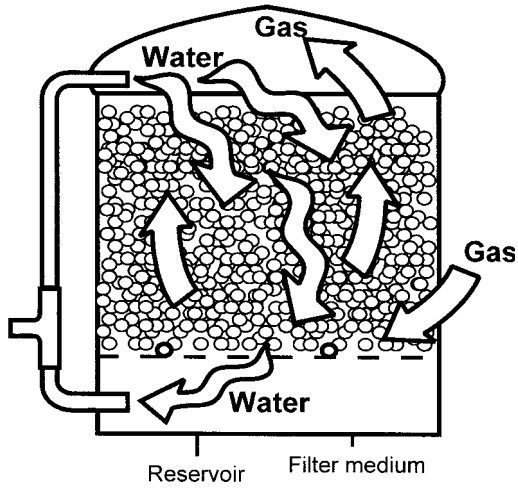

Figure 4.3 *Biotrickling filter*

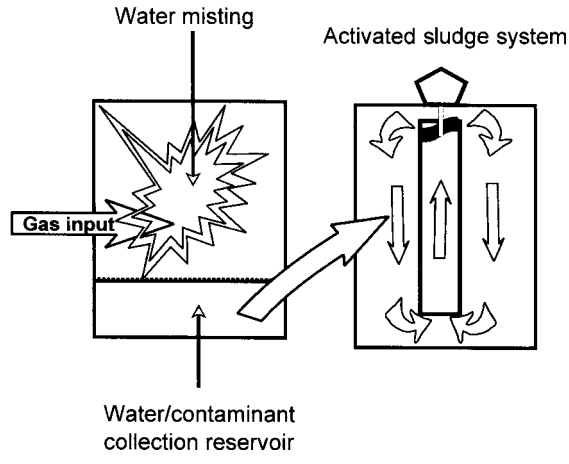

Figure 4.4 *Bioscrubber*

Bioscrubbers

Although it is normally included in the same group, the bioscrubber (Figure 4.4) is not itself truly a biological treatment system, but rather a highly efficient method of removing odour components by dissolving them. Unsurprisingly, then, it is most appropriate for hydrophilic compounds like acetone or methanol.

The gas to be treated passes through a fine water spray generated as a mist or curtain within the body of the bioscrubber vessel. The contaminant is absorbed into the water, which subsequently pools to form a reservoir at the bottom. The contaminant solution is then removed to a secondary bioreactor where the actual process of biodegradation takes place. In practice, activated sludge systems (which are described in detail in Chapter 6) are often used in this role.

As in the preceding case, process control can be achieved by monitoring the water phase and adding nutrients, buffers or fresh water as appropriate.

Other options

It is important to be aware that biotechnology is not the only answer to controlling air pollution. A number of alternative approaches exist, though it is clearly beyond the scope of this book to discuss them at length. The following brief outline may help to give a flavour of the wider context, but to understand how the various technologies compare, the reader should seek more detailed information.

Absorption

Absorbing the compound in a suitable liquid; this may oxidise or neutralise it in the process.

Adsorption

Activated carbon preferentially adsorbs organic molecules; this can be tailored to give contaminant-specific optimum performance.

Incineration

High temperature oxidation; effective against most contaminants, but costly.

Ozonation

Use of ozone to oxidise some contaminants, like hydrogen sulphide; effective but can be costly.

The main advantages of biotechnological approaches to the issue of air contamination can be summarised as:

- competitive capital costs;
- low running costs;
- low maintenance costs;
- low noise;
- no carbon monoxide production;
- avoids high temperature requirement or explosion risk;
- safe processes with highly 'green' profile;
- robust and tolerant of fluctuation.

As was discussed in the first chapter, pollution control stands as one of the three major intervention points for the application of environmental biotechnology. Having defined some of the major principles and issues, the next chapter will examine how they are addressed in practice. However, it must not be forgotten that, as with all tripods, each leg is equally important; the potential contribution to be made by the so-called 'clean technologies' in manufacturing should not be overlooked. Much of the focus of environmental biotechnology centres on the remediation of pollution or the treatment of waste products. In many respects, this tends to form the natural constituency of the science and is, certainly, where the bulk of practical applications have generally occurred. While the benefits of the controlled biodegradation of unwanted wastes or contaminants is clear, this does typify 'end-of-pipe' thinking and has led, to some extent justifiably, to the criticism that it merely represents moving the problem from one place to another. Another option to deal with both these ongoing problems is, simply, to avoid their production in the first place and while this may seem over-idealistic in some aspects, it does have a clear and logical appeal. Throughout this book, 'environmental' biotechnology is defined in the broad sense of the utilisation of applied biological methods to the benefit of the environment. Thus, any use of the life sciences which removes, remediates or obviates contamination of the biosphere falls firmly within its remit and *a priori* action, to avoid the problem in

the first place must be preferential. The proverbial ounce of prevention is worth a pound of cure.

The current emphasis on clean-up and treatment is largely the result of historical circumstance. As legislation has become more stringent, the regulation of waste and pollution has correspondingly forced the pace of environmental intervention. In addition, the prevalence of 'the polluter pays' principle, coupled with ever greater pressures to redevelop existing 'brown-field' sites, in preference to *de novo* development has inevitably necessitated a somewhat reactive response. However, increasingly biotechnologies are being developed which, though perhaps not 'environmental' in themselves, bring significant benefits to this sphere. Their advantages to industry in terms of reduced demands for integrated pollution control and minimised waste disposal costs also suggest a clear likelihood of their success in the commercial sphere. Generally, the environment has tended to fare best when its interests and economic ones go hand in hand and the pre-emptive approach which the new technologies herald seems ideally suited to both.

'Clean' Technology

The mechanisms by which pollution or waste may be reduced at source are varied. They may involve changes in technology or processes, alteration in the raw materials used or a complete restructuring of procedures. Generally speaking, biotechnological interventions are principally limited to the former aspects, though they may also prove instrumental in permitting procedural change. The main areas in which biological means may be relevant fall into three broad categories:

- process changes;
- biological control;
- bio-substitutions.

In the following discussions of these three groups, it is not suggested that the examples cited are either comprehensive or exhaustive; they are simply intended to illustrate the wide potential scope of applications open to biotechnology in clean manufacturing. For precisely the reasons mentioned in respect of the economic aspects of this particular area of industrial activity, the field is a fast evolving one and many more types of biotechnological interventions are likely in the future, especially where commercial pressures derive a competitive advantage.

Process Changes

Replacement of existing chemical methods of production with those based on microbial or enzyme action is an important potential area of primary pollution prevention and is one role in which the use of genetically modified organisms

could give rise to significant environmental benefit. Biological synthesis, either by whole organisms or by isolated enzymes, tends to operate at lower temperatures and, as a result of high enzymatic specificity, gives a much purer yield with fewer byproducts, thus saving the additional cost of further purification. There are many examples of this kind of industrial usage of biotechnology. In the cosmetics sector, there is a high demand for isopropyl myristate which is used in moisturising creams. The conventional method for its manufacture has a large energy requirement, since the process runs at high temperature and pressure to give a product which needs further refinement before it is suitable for use. An alternative approach, using enzyme-based esterification offers a way to reduce the overall environmental impact by deriving a cleaner, odour-free product, and at higher yields, with lower energy requirements and less waste for disposal.

Textile industry

There is a long tradition of the use of biological treatment methods in the clothing and textile industry, dating back to the first use of amylase enzymes from malt extract, at the end of the nineteenth century, to degrade starch-based sizes for cheap and effective reduction of fabric stiffness and improvement to its drape. Currently, novel enyzmatic methods provide a fast and inexpensive alternative to traditional flax extraction by breaking down the woody material in flax straw, reducing the process time from seven to ten days, down to a matter of hours. The enzyme-based retting processes available for use on hemp and flax produce finer, cleaner fibres, and, consequently, novel processing techniques are being developed to take advantage of this. Interest is growing in the production of new, biodegradable polymeric fibres which can be synthesised using modified soil bacteria, avoiding the current persistence of these materials in landfills, long after garments made from them are worn out.

In natural fibre production enzymes are useful to remove the lubricants which are introduced to prevent snagging and reduce thread breakage during spinning, and to clean the natural sticky secretions present on silk. The process of bioscouring for wool and cotton, uses enzymes to remove dirt rather than traditional chemical treatments and bio-bleaching uses them to fade materials, avoiding both the use of caustic agents and the concomitant effluent treatment problems such conventional methods entail. Biological catalysts have also proved effective in shrink-proofing wool, improving quality while ameliorating the wastewater produced, and reducing its treatment costs, compared with chemical means.

A process which has come to be called biopolishing involves enzymes in shearing off cotton microfibres to improve the material's softness and the drape and resistance to pilling of the eventual garments produced.

Biostoning has been widely adopted to produce 'stone-washed' denim, with enzymes being used to fade the fabric rather than the original pumice stone method, which had a higher water consumption and caused abrasion to the denim.

However, perhaps the most fitting example of environmental biotechnology in the textile industry, though not really in a 'clean technology' role, is the incorporation of adsorbers and microbes within a geotextile produced for use in land management around railways. Soaking up and subsequently biodegrading diesel and grease, the textile directly reduces ground pollution, while also providing safer working conditions for track maintenance gangs and reducing the risk of fire.

Leather industry

The leather industry has a lengthy history of using enzymes. In the bating process, residual hair and epidermis, together with nonstructural proteins and carbohydrates, are removed from the skins, leaving the hide clean, smooth and soft. Traditionally, pancreatic enzymes were employed. Moreover, something in the region of 60% of the input raw materials in leather manufacturing ultimately ends up being discarded and enzyme additions have long been used to help manage this waste. Recent advances in biotechnology have seen the upsurge in the use of microbially-derived biological catalysts, which are cheaper and easier to produce, for the former applications, and the possibility of converting waste products into saleable commodities, in the latter.

As well as these improvements on existing uses of biotechnology, new areas of clean application are emerging for tanners. Chemical methods for unhairing hides dissolve the hairs, making for efficient removal, but adding to the treatment cost, and the environmental implications, of the effluents produced, which are of high levels of COD and suspended solids. Combining chemical agents and biological catalysts significantly lessens the process time while reducing the quantities of water and chemicals used. The enzymes also help make intact hair recovery a possibility, opening up the prospect of additional income from a current waste. It has been estimated that, in the UK, for a yearly throughput of 400 000 hides, enzymatic unhairing offers a reduction of around 2% of the total annual running costs (BioWise 2001). While this may not seem an enormous contribution, two extra factors must be borne in mind. Firstly, the leather industry is very competitive and, secondly, as effluent treatment becomes increasingly more regulated and expensive, the use of clean manufacturing biotechnology will inevitably make that margin greater.

Degreasing procedures are another area where biotechnological advances can benefit both production and the environment, since conventional treatments produce both airborne volatile organic compounds (VOCs) and surfactants. The use of enzymes in this role not only gives better results, with a more consistent quality, better final colour and superior dye uptake, but also considerably reduces VOC and surfactant levels. The leather industry is also one of the places where biosensors may have a role to play. With the ability to give almost instantaneous detection of specific contaminants, they may prove of value in giving early warning of potential pollution problems by monitoring production processes as they occur.

Desulphurisation of coal and oil

Microbial desulphurisation of coal and oil represents a further potential example of pollution control by the use of clean technology. The sulphur content of these fossil fuels is of environmental concern principally as a result of its having been implicated in the production of acid rain, since it produces sulphur dioxide (SO_2) on combustion. Most of the work done to date has tended to focus on coal, largely as a result of its widespread use in power stations, though similar worries equally surround the use of high-sulphur oils, particularly as the reserves of low-sulphur fuels dwindle. The sulphurous component of coal typically constitutes between 1–5%; the content for oil is much more variable, dependent on its type and original source.

There are two main ways to reduce SO_2 emissions. The first is to lessen the sulphur content of the fuel in the first place, while the second involves removing it from the flue gas. There are a number of conventional methods for achieving the latter, the most commonly encountered being wet scrubbing, though a dry absorbent injection process is under development. At present, the alternative approach of reducing the sulphur present in the initial fuel, works out around five times more expensive than removing the pollutant from the flue gas, though as stock depletion forces higher sulphur coals and oils to be burnt, the economics of this will start to swing the other way. Methods for achieving a sulphur content reduction include washing pulverised coal and the use of fluidised bed technology in the actual combustion itself, to maximise clean burn efficiency.

Sulphur is present in coal in a variety of different forms, both organic and inorganic and biological methods for its removal have been suggested as alternatives to the physical means mentioned above. Aerobic, acidophilic chemolithotrophes like certain of the *Thiobacillus* species, have been studied in relation to the desulphurisation of the inorganic sulphur in coal (Rai 1985). Microbes of this genus have long been known to oxidise sulphur during the leaching of metals like copper, nickel, zinc and uranium from low grade sulphide ores. Accordingly, one possible application which has been suggested would be the use of a heap-leaching approach to microbial desulphurisation at the mine itself, which is a technique commonly employed for metals. However, though this is, clearly, a cheap and simple solution, in practice it is difficult to maintain optimum conditions for the process. The micro-organisms which have most commonly been used to investigate this possible approach are mesophiles and the rapid temperature increases experienced coupled with the lengthy period of contact time required, at around 4–5 days, form major limiting factors. The use of extreme thermophile microbes, like *Sulfolobus sp.* may offer the way ahead, giving a faster rate of reaction, though demanding the more sophisticated and engineered environment of a bioreactor if they are to achieve their full process efficiency.

The removal of organic sulphur from coal has been investigated by using model organic substrates, most commonly dibenzothiophene (DBT). In laboratory experiments, a number of organisms have been shown to be able to remove

organic sulphur, including heterotrophes (Rai and Reyniers 1988) like *Pseudomonas*, *Rhizobium* and the fungi *Paecilomyces* and chemolithotrophes like *Sulfolobus*, mentioned earlier. These all act aerobically, but there is evidence to suggest that some microbes, like *Desulfovibrio* can employ an anaerobic route (Holland *et al*. 1986). While the use of such model substrates has some validity, since thiophenes are the major organic sulphur components in coal, how well their breakdown accurately reflects the situation for the real material remains much less well known.

A range of putative bioreactor designs for desulphurisation have been put forward, involving treatment systems of varying complexity, which may ultimately provide an economic and efficient method for removing sulphur from these fuels prior to burning. However, the state of the art is little advanced beyond the laboratory bench and so the benefits of large-scale commercial applications remain to be seen.

Biological Control

The use of insecticides and herbicides, particularly in the context of agricultural usage, has been responsible for a number of instances of pollution and many of the chemicals implicated are highly persistent in the environment. Though there has been a generalised swing away from high dosage chemicals and a widespread reduction in the use of recalcitrant pesticides, worldwide there remains a huge market for this class of agrochemicals. As a result, this is one of the areas where biotechnological applications may have significant environmental impact, by providing appreciably less damaging methods of pest management. The whole concept of biological control took a severe blow after the widely reported, disastrous outcome of Australia's attempts to use the Cane Toad (*Bufo marinus*) to control the cane beetle. However, in principle, the idea remains sound and considerable research effort has gone into designing biological systems to counter the threat of pests and pathogens. Some of these, in respect of soil-borne plant pathogens and biopesticides, are discussed elsewhere in this work and, accordingly, do not warrant lengthy reiteration here.

The essence of the specifically environmental contribution of this type of biointervention lies in its ability to obviate the need for the use of polluting chemicals and, consequently, leads to a significant reduction in the resultant instances of contamination of groundwater or land. However, one of the major limitations on the effective use of biocontrols is that these measures tend to act more slowly than direct chemical attacks and this has often restricted their use on commercial crops. In fairness, it must be clearly stated that biotechnology *per se* is not a central, or even necessary, requirement for all of biological control, as many methods rely on whole organism predators, which, obviously, has far more bearing on an understanding of the ecological interactions within the local environment. However, the potential applications of biotechnology to aspects of pest/pathogen/organism

dynamics, as examined in other sections of this book, has a supportive role to play in the overall management regime and, thus, there exists an environmental dimension to its general use in this context.

Biological control methods can provide an effective way to mitigate pesticide use and thus the risk represented to the environment and to public health. In addition, unlike most insecticides, biocontrols are often highly target-specific reducing the danger to other nonpest species. Against this, biological measures typically demand much more intensive management and careful planning than the simple application of chemical agents. Success is much more dependent on a thorough understanding of the life-cycles of the organisms involved and can often be much more of a long-term project. In addition, though high specificity is, generally, a major advantage of biocontrol measures, under some circumstances, if exactly the right measure is not put in place, it may also permit certain pests to continue their harmful activities unabated. Considering the huge preponderance of insect species in the world, a large number of which pose a threat to crops or other commodities and thus represent an economic concern, it is small wonder that the global insecticide market has been estimated at over $8 billion (US) per year. Accordingly, much of the biological control currently in practice relates to this group of animals.

Whole-organism approaches

There are three main ways in which whole-organism biological pest control may be brought about. Classical biological control, as with the previously mentioned Cane Toad, requires the importation of natural predators and is principally of use when the pest in question is newly arrived in an area, often from another region or country, having left these normal biological checks behind. Another form of control involves conservation measures aimed at bolstering the predatory species, which may be a valuable approach when natural enemies already exist within the pest's range. However, the third method, augmentation, is more relevant to the concepts of biotechnology and refers to means designed to bring about the increase in effectiveness of natural enemies to a given pest. This may consist simply of artificially rearing them in large numbers for timed release or may extend to more intensive and sophisticated measures like the modification, either by selective breeding or genetic manipulation, of the predator such that it is better able to locate or attack the pest.

One attempt at augmentation which has been tried commercially is the production of parasitic nematodes. Juvenile stages of the nematodes, which are then only around 500 μm long and 20 μm wide, can enter soil insects and many carry pathogenic bacteria in their guts. Once ingested, these bacteria pass out of the nematode and multiply inside the insect, typically causing death within a few days. Five species of nematode were made available on the US agricultural market, namely *Steinernema carpocapsae*, *S. riobravis*, *S. feltiae*, *Heterorhabditis bacteriophora* and *H. megidis*, each being effective against different groups of

insects. Despite much research and development effort, the results were largely unpredictable, with success against many of the target species, like wireworms and root maggots, proving elusive. One avenue of potential application for this technology may, however, lie in the control of cockroaches, which have been found to be the most vulnerable species to augmented nematode attack (Georgis 1996). However, there still remain some technical problems to overcome in terms of ensuring a level of parasite delivery before widespread uptake is likely. Augmentation is, obviously, a highly interventionist approach and relies on a regime of continual management to ensure its effectiveness.

There is also a role for the engineered application of biologically derived chemicals in this sector. One example of this is the growing interest in *Azadirachta indica*, the neem, a plant which is found naturally in over 50 countries around the world including India, where its medicinal and agricultural value has been known for centuries. The compound azadirachtin has been identified and isolated from the plant and it has been shown to have broad spectrum insecticidal properties, acting to disturb larval moults and preventing metamorphosis to the imago. Additionally, it also seems to repel many leaf-eating species, and trials involving the direct foliar application of azadirachtin has shown it to be an effective way of protecting crop plants (Georgis 1996). This duality of action makes it a particularly appealing prospect for wide-scale applications, if suitable methods for its production can be made commercially viable.

Semiochemical agents

However, perhaps one of the best examples of the use of such biological technologies in pest control is the development of isolated or synthesised semiochemical agents.

Semiochemicals are natural messenger substances which influence growth, development or behaviour in numerous plant and animal species and include the group known as pheromones, a number of which are responsible for sexual attraction in many insects. This has been successfully applied to control various forms of insect pests, either directly to divert them from crops and trap them, or indirectly to trap their natural enemies in large numbers for introduction into the fields for defence.

For example, crops worldwide suffer severe damage as a result of a number of pentatomid insects, amongst which are several of the common brown stink bugs of North America (*Euschistus* spp.). They arrive late in the growing season and often cause major harm before detection. A major part of biocontrol involves obtaining a thorough understanding of their migration patterns and to help achieve it in this case, a pheromone, methyl 2E,4Z-decadienoate, has been produced commercially to aid trapping. The early success of this is being developed to extend its scope in three main directions. Firstly, to capture and eliminate the pests themselves, secondly, to harvest predatory stink bugs for bioaugmentative control

programmes and thirdly, to identify more pheromones to widen the number of phytophagous stink bug species which can be countered in this way.

As something of an aside, one interesting and somewhat unusual use has been proposed for this technology. The Siberian moth *Dendrolimus superans* is a vigorous defoliating pest of northern Asian coniferous forests and, though it does not presently occur in North America, its arrival is much feared. In an attempt to provide a first line of defence against this potential threat to native woodlands, it has been suggested that a blend of Z5,E7-dodecadienol and Z5,E7-dodecadienol, which has been shown to act as a powerful sex attractant for male Siberian moths, be deployed at US ports of entry.

However, as illustrated by the case of another pentatomid, *Nezara viridula*, the southern green stink bug, the use of this approach to biological control is not universally applicable. These insects are major agricultural pests affecting a variety of field crops, vegetables, fruits and nuts. While it has been known for sometime that sexually mature males produce an attractant pheromone, the active ingredients of which have been identified, early attempts to use this knowledge to exclude them from crops have been of only limited effectiveness. As a result, an alternative method of Nezara control has been suggested involving the genetic engineering of its gut symbionts to produce a reduced tolerance of environmental stress. Preliminary work at the Agricultural Research Center, Beltsville, USA has isolated and cultured *in vitro* a gram-negative bacterium from the mid-gut of the pest insect, which appears to be a specific symbiont and has been putatively identified as a species of *Yokenella*. This kind of application of transgenic technology may increasingly be the future of biological control for species which do not respond favourably to pheromone trapping.

Not all approaches to biocontrol truly qualify as environmental biotechnologies, at least not within the frame of reference used in this book. However, where the use of biological systems results in reduced insecticide use and thus a corresponding lowering of the attendant pollution potential, the net environmental gains of the application of biotechnology are clear.

Biosubstitutions

The biosubstitution of suitable, less harmful alternatives for many of today's polluting substances or materials is a major potential avenue for the environmentally beneficial application of biotechnology. The question of biofuels and the major renewable contribution which organised, large-scale biomass utilisation could make to energy demands is examined in some detail in Chapter 10 and will not, therefore be repeated here. The biological production of polymers, likewise, features in the same section on integrated biotechnology and, though clearly distinctly germane to the present discussion, will also not appear in this consideration. However, the other major use of mineral oils, as lubricants, is an excellent case study of the opportunities, and obstacles, surrounding biotech

substitutes. Biodegradable alternatives to traditional lubricating oils have existed for some time, but in many ways they exemplify the pressures which work against novel biological products.

Barriers to uptake

Typically, most of the barriers which they must overcome are nontechnical. The pollution of many inland and coastal waters around the world is a well-appreciated environmental problem and wider use of these nontoxic, readily biodegradable alternatives products could make a huge difference. The main obstacles to wider market acceptance of the current generation of alternative lubricants are neither performance based, nor rooted in industrial conservatism. Cost is a major issue, as biolubricants are around twice as expensive as their conventional equivalents, while for some more specialist formulations the difference is significantly greater. Though, inevitably, users need to be convinced of the deliverable commercial benefits, the potential market is enormous. The petrochemical industry has sought to meet the growing demand for more environmentally friendly products by developing biodegradable lubricants based on crude oil. However, with the agricultural sector, particularly throughout Europe, being encouraged to grow nonfood crops commercially, there is a clear opportunity for a sizeable vegetable oil industry to develop, though the attitude of heavy industry will prove crucial.

While there is no denying the burgeoning interest in biolubricants, the actual machinery to be lubricated is extremely expensive, and enforced downtime can be very costly. Understandably as a result, few equipment operators are willing to risk trying these new, substitute oils, as original equipment manufacturers (OEMs) are seldom willing to guarantee their performance, not least because vegetable products are often wrongly viewed as inferior to traditional oils.

Simple biosubstitutions

Not all biosubstitutions need be the result of lengthy chemical or biochemical synthesis or processing and far simpler forms of biological production may provide major environmental benefits. The production of biomass fuels for direct combustion under short rotation coppicing management, described in Chapter 10, is one example. The use of what have been termed ‘eco-building materials’ formed from hemp, hay, straw and flax and then compressed, as an ecological alternative to conventional materials in the construction industry, is another.

Traditional building approaches have a number of broadly environmental problems. Adequate soundproofing, particularly in home or work settings where traffic, industrial or other noises are a major intrusive nuisance can be difficult or costly to achieve for many standard materials. Walls made from eco-materials have been found to be particularly effective at sound suppression in a variety of applications, including airports, largely due to a combination of the intrinsic

natural properties of the raw materials and the compression involved in their fabrication. In a number of trials of these materials, principally in Austria, where they originated, eco-walls have consistently been shown to provide significant improvements in the quality of living and working conditions. In addition, construction and demolition waste, consisting of concrete rubble, timber fragments, brick shards and the like, poses a considerable disposal problem for the industry, particularly with increasingly stringent environmental regulation and rising storage and landfill costs. Though various recycling initiatives and professional codes of practice have helped ease the situation, there is an obvious advantage in a relatively inexpensive, lightweight and sustainable material which is truly biodegradable. At present, the use of this technology has been limited to small-scale demonstrations, though wider uptake is currently being promoted through the European Union's Innovation Relay Centre network. The appeal of this, and other biological materials production methods for use in construction, the automotive and aerospace industries is clear, but it is very early days in their development. How successful they will ultimately prove to be remains to be seen.

Closing Remarks

As this chapter has shown, pollution and its mitigation have major ramifications in many diverse fields both for industry and in the wider sphere of general human activities. The potential contributions of clean technologies discussed in the final section have enormous bearing on the reduction of contamination *ab initio*, and, clearly, avoiding a problem in the first place is far better than cleaning it up after it has occurred. However, in most cases, current applications of environmental biotechnology to treat pollutants and wastes far outnumber the practical examples of clean biomanufacturing and so the rest of this book will address this more common use.

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Case Study 4.1 Microbial Pollution Control (Maine, USA)

Pollution control often involves either minimising existing problems or dealing with their aftermath and biological treatment can frequently be a very cost-effective option in either case.

Caldwell Environmental of Acton, USA have developed a number of proprietary biological approaches to deal with environmental contamination. One of the most successful is BioRem® ST, an affordable microbial process designed to liquefy the solid waste build up in septic tanks, keeping the system in optimum working order, while odour problems are also largely eliminated. Routine applications also help to prevent drain blockage, which is often a problem for these installations. The formulation uses Class 1 classified bacteria, as defined by the American Type Culture Collection, which are safe to humans, animal and plant species, and approved for use in federally inspected meat and poultry processing plants. Able to reduce nitrogen and break down fats, carbohydrates, starches, oils, greases and detergents, the treatment offers an effective alternative to reliance on corrosive or toxic chemicals.

A good example of its large-scale use is the clean-up of accidental pollution from a food market in Maine, USA. The facility, which was located less than 75 metres (250 feet) from a lake, had a 250 litre (1000 gallon) septic tank. Unbeknown to the owners, the soakaway zone had failed and the discharge was going directly into the water body, this fact only coming to light during a routine inspection by the local authorities, using a dye tracer. Subsequent pollution control began with the daily dosing of the soakaway zone with the bacterial formulation, while controlled levels of additional nutrients were pumped in to bioenhance the area, thus optimising microbial action. Nine months later, the treatment was complete, and the necessary operational certifications granted for its continued use.

The value and effectiveness of this intervention would appear to have been more than adequately demonstrated, since the site owners have instigated an ongoing preventative maintenance regime, involving continued systematic microbial dosing, to avoid any repetition in the future.

5

Contaminated Land and Bioremediation

Contaminated land is another example of a widely appreciated, yet often poorly understood, environmental problem, in much the same way as discussed for pollution in the last chapter. That this should be the case is, of course, unsurprising, since the two things are intimately linked, the one being, in essence, simply the manifestation of the other. The importance of land remediation in cleaning up the residual effects of previous human activities on a site lies in two spheres. Firstly, throughout the world, environmental legislation is becoming increasingly stringent and the tightening up of the entire regulatory framework has led to both a real drive for compliance and a much greater awareness of liability issues within industry. Secondly, as the pressure grows to redevelop old, unused or derelict so-called 'brown-field' sites, rather than develop previously untouched 'green-field', the need to remove any legacy of previous occupation is clear. A number of technologies are available to achieve such a clean-up, of which bioremediation, in its many individual forms, is only one. Though it will, of course, provide the main focus of this discussion, it is important to realise that the arguments presented elsewhere in this book regarding the high degree of specificity which governs technology selection *within* biotechnological applications also applies *between* alternative solutions. In this way, for some instances of contamination, expressly nonbiological methods of remediation may be indicated as the best practicable environmental option (BPEO). It is impossible to disassociate contextual factors from wider issues entirely. Accordingly, and to establish the relevancy of the wider setting, alternative remediation techniques will be referred to a little later in this chapter.

The idea of 'contaminated land' is something which is readily understood, yet, like pollution, somewhat more difficult to define absolutely. Implicit is the presence of substances which, when present in sufficient quantity or concentration, are likely to cause harm to the environment or human health. Many kinds of sites may give rise to possible contamination concerns, such as asbestos works, chemical works, garages and service stations, gas works, incinerators, iron and steel works, metal fabrication shops, paper mills, tanneries, textile plants, timber treatment plants, railway yards and waste disposal sites. This list is not, of course, exhaustive and it has been estimated that in the UK alone something in the region

of 360 000 hectares (900 000 acres) of land may be affected by contamination in one form or another (BioWise 2001). Much of this will, of course, be in prime urban locations, and therefore has the potential to command a high market price, once cleaned up.

Since the whole question of contaminated land increasingly forms the basis of law and various professional codes of practice, there is an obvious need for a more codified, legal definition. The version offered in Section 57 of the UK Environment Act 1995 is a typical example:

> any land which appears. . . . .to be in a condition that. . . . .significant harm is being caused or there is a significant possibility of significant harm. . . . (or). . . . .pollution of controlled waters.

In this, *harm* is expressly defined as to *human health, environment, property*.

As was mentioned earlier, land remediation continues to grow in importance because of pressures on industry and developers. The motive force is, then, a largely commercial one and, consequently, this imposes its own set of conditions and constraints. Much of environmental biotechnology centres on the 'unwanted' aspects of human activity and the clean-up of contaminated land is no exception to this general trend. As such, it is motivated by necessity and remedies are normally sought only when and where there is unacceptable risk to human health, the environment and occasionally to other vulnerable targets. In broad terms it is possible to view the driving forces on remediation as characterised by a need to limit present or future liability, increase a site's value, ease the way for a sale or transfer, comply with legislative, licensing or planning requirements, or to bolster corporate image or public relations. Generally, one or more of these have to be present before remediation happens.

Having established the need for treatment, the actual remedies to be employed will be based on a realistic set of priorities and will be related to the risk posed. This, of course, will require adequate investigation and risk assessment to determine. It is also important to remember in this context that, since the move to remediate is essentially commercial, only land for which remediation is either necessary or worthwhile will tend to be treated and then to a level which either makes it suitable for its intended use or brings it to a condition which no longer poses an unacceptable risk.

It should be apparent, then, from the preceding discussion that the economics of remediation and the effective use of resources are key factors in the whole contaminated land issue. Hence, in purely economic terms, remediation will only take place when one or more of the driving forces becomes sufficiently compelling to make it unavoidable. It will also tend towards the minimum acceptable standard necessary to achieve the required clean-up. This is not an example of industrial self-interest at its worst, but rather the exercise of responsible management, since resources for remediation are typically limited and so their effective use is of great importance. To 'over' remediate any one given site could seriously

compromise a company's ability to channel sufficient funds to deal with others. The goal of treating land is to make it suitable for a particular purpose or so that it no longer poses unacceptable risk and, once the relevant aim has been achieved, further treatment is typically not a good use of these resources. Generally it would be judged better to devote them to cleaning up other sites, which maximises the potential reuse of former industrial land thereby protecting urban open spaces and the countryside from development pressure. In the long term, the sustainable use of land largely depends on making sure that it is maintained at a level which enables its continued best use for its current or intended purpose. In this respect, discussions of absolute quality become less relevant than a consideration of minimum acceptable standards.

The choice of method and the determination of the final remediation standard will always be chiefly governed by site-specific factors including intended use, local conditions and sensitivities, potential risk and available timeframe. For this reason, it is appropriate to take a brief overview of the available technologies at this point, to set the backdrop for the discussions of the specifically biotechnological methods to come.

Remediation Methods

The currently available processes for soil remediation can be divided into five generalised categories:

- biological;
- chemical;
- physical;
- solidification/vitrification;
- thermal.

Biological

Biological methods involve the transformation or mineralisation of contaminants to less toxic, more mobile, or more toxic but less mobile, forms. This can include fixation or accumulation in harvestable biomass crops, though this approach is discussed more fully later in Chapter 7.

The main advantages of these methods are their ability to destroy a wide range of organic compounds, their potential benefit to soil structure and fertility and their generally nontoxic, 'green' image. On the other hand, the process end-point can be uncertain and difficult to gauge, the treatment itself may be slow and not all contaminants are conducive to treatment by biological means.

Chemical

Toxic compounds are destroyed, fixed or neutralised by chemical reaction. The principal advantages are that under this approach, the destruction of biologically

recalcitrant chemicals is possible and toxic substances can be chemically converted to either more or less biologically available ones, whichever is required. On the downside, it is possible for contaminants to be incompletely treated, the reagents necessary may themselves cause damage to the soil and often there is a need for some form of additional secondary treatment.

Physical

This involves the physical removal of contaminated materials, often by concentration and excavation, for further treatment or disposal. As such, it is not truly remediation, though the net result is still effectively a clean-up of the affected site. Landfill tax and escalating costs of special waste disposal have made remediation an increasingly cost-effective option, reversing earlier trends which tended to favour this method. The fact that it is purely physical with no reagent addition may be viewed as an advantage for some applications and the concentration of contaminants significantly reduces the risk of secondary contamination. However, the contaminants are not destroyed, the concentration achieved inevitably requires containment measures and further treatment of some kind is typically required.

Solidification/vitrification

Solidification is the encapsulation of contaminants within a monolithic solid of high structural integrity, with or without associated chemical fixation, when it is then termed 'stabilisation'. Vitrification uses high temperatures to fuse contaminated materials.

One major advantage is that toxic elements and/or compounds which cannot be destroyed, are rendered unavailable to the environment. As a secondary benefit, solidified soils can stabilise sites for future construction work. Nevertheless, the contaminants are not actually destroyed and the soil structure is irrevocably damaged. Moreover, significant amounts of reagents are required and it is generally not suitable for organic contaminants.

Thermal

Contaminants are destroyed by a heat treatment, using incineration, gasification, pyrolysis or volatisation processes. Clearly, the principal advantage of this approach is that the contaminants are most effectively destroyed. On the negative side, however, this is achieved at typically very high energy cost, and the approach is unsuitable for most toxic elements, not least because of the strong potential for the generation of new pollutants. In addition, soil organic matter, and, thus, at least some of the soil structure itself, is destroyed.

In Situ and *Ex Situ* Techniques

A common way in which all forms of remediation are often characterised is as *in situ* or *ex situ* approaches. These represent largely artificial classes, based

on no more than where the treatment takes place – on the site or off it – but since the techniques within each do share certain fundamental operational similarities, the classification has some merit. Accordingly, and since the division is widely understood within the industry, these terms will be used within the present discussion.

In situ

The major benefit of approaches which leave the soil where it is for treatment, is the low site disturbance that this represents, which enables existing buildings and features to remain undisturbed, in many cases. They also avoid many of the potential delays with methods requiring excavation and removal, while additionally reducing the risk of spreading contamination and the likelihood of exposing workers to volatiles. Generally speaking, *in situ* methods are suited to instances where the contamination is widespread throughout, and often at some depth within, a site, and of low to medium concentration. Additionally, since they are relatively slow to act, they are of most use when the available time for treatment is not restricted.

These methods are not, however, without their disadvantages and chief amongst them is the stringent requirement for thorough site investigation and survey, almost invariably demanding a high level of resources by way of both desktop and intrusive methods. In addition, since reaction conditions are not readily controlled, the supposed process 'optimisation' may, in practice, be less than optimum and the true end-point may be difficult to determine. Finally, it is inescapable that all site monitoring has an in-built time lag and is heavily protocol dependent.

Ex situ

The main characteristic of *ex situ* methods is that the soil is removed from where it originally lay, for treatment. Strictly speaking this description applies whether the material is taken to another venue for clean-up, or simply to another part of the same site. The main benefits are that the conditions are more readily optimised, process control is easier to maintain and monitoring is more accurate and simpler to achieve. In addition, the introduction of specialist organisms, on those occasions when they may be required, is easier and/or safer and generally these approaches tend to be faster than corresponding *in situ* techniques. They are best suited to instances of relatively localised pollution within a site, typically in 'hot-spots' of medium to relatively high concentration which are fairly near to the surface.

Amongst the main disadvantages are the additional transport costs and the inevitably increased likelihood of spillage, or potential secondary pollution, represented by such movement. Obviously these approaches require a supplementary area of land for treatment and hence they are typically more expensive options.

As Figure 5.1 illustrates, the decision to use *in situ* or *ex situ* techniques is a comparatively straightforward 'black-or-white' issue at the extremes for either

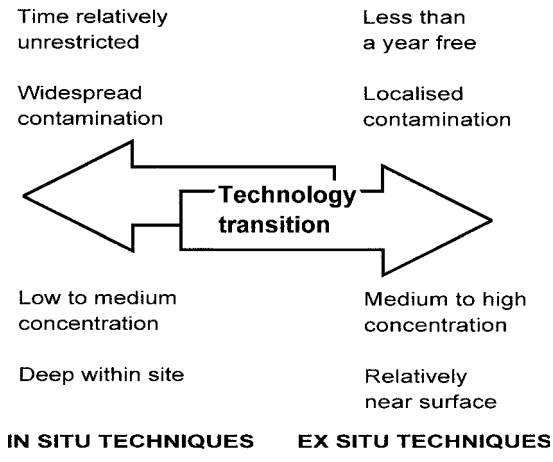

Figure 5.1 *Factors affecting technology suitability*

option. However, the middle ground between them comprises many more shades of grey, and the ultimate resolution in these cases is, again, largely dependent on individual circumstance.

Intensive and Extensive Technologies

Though the *in situ/ex situ* classification has established historic precedence, of recent times an alternative approach to categorise remediation activities has emerged, which has not yet achieved the same widespread recognition or acceptance, but does, nevertheless offer certain advantages over the earlier approach. Perhaps the most significant of these is that it is a more natural division, based on genuine similarities between technologies in each class. Thus the descriptions 'intensive' and 'extensive' have been suggested.

Intensive technologies can be characterised as sophisticated, fast-acting, high intervention strategies, with a heavy demand for resources and high initiation, running and support costs. Their key factors are a fast response and low treatment time, which makes them excellent for heavy contamination conditions, since they can make an immediate lessening in pollutant impact. Soil washing and thermal treatments are good examples of 'intensive' approaches.

Extensive methods are lower-level interventions, typically slower acting, based on simpler technology and less sophisticated engineering, with a smaller resource requirement and lower initiation, running and support costs. These technologies have a slower response and a higher treatment time, but their lower costs make wider application possible, particularly since extensive land remediation treatments do less damage to soil quality. Accordingly, they are well suited to large-scale treatment where speed is not of the essence. Examples include

composting, the promotion of biological activity *in situ* within the root-zone, precipitation of metal sulphides under anaerobic conditions and the cropping of heavy metal accumulator plants.

All these systems of classification are at best generalisations, and each can be useful at different times, dependent on the purpose of the consideration. They are merely a convenient way of looking at the available techniques and should not be regarded as anything more than a helpful guide. As a final aspect of this, it is possible to examine the various forms of land remediation technologies in terms of their overall functional principle. Hence, the approaches may be categorised as 'destructive', 'separating' or 'containing', dependent on their fundamental mode of operation, as Figure 5.2 illustrates. The principal attraction of this systemisation is that it is defined on the basis of representing the fate of the pollutant,

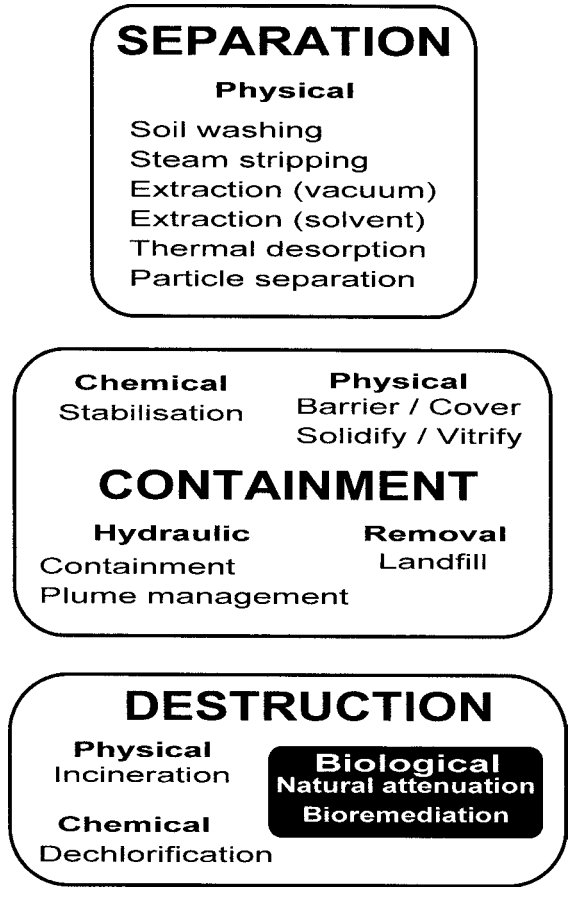

Figure 5.2 *Technology classification*

rather than the geographical location of the work or the level of complexity of the technology used, as in the previous cases. In addition, it can also be relatively easily extended to take account of any given technology.

Process Integration

However they are classified, the fact remains that all the individual technologies available each have their limitations. As a result, one potential means of enhancing remediation effectiveness which has received increasing attention is the use of a combination approach, integrating different processes to provide an overall treatment. The widespread application of this originated in the USA and the related terms used to describe it, 'bundled technologies' or 'treatment trains' have quickly become commonly used elsewhere. The goal of process integration can be achieved by combining both different fundamental technologies (e.g. biological and chemical) and sequences of *in situ* or *ex situ*, *intensive* or *extensive* regimes of processing. In many respects, such a 'pick-and-mix' attitude makes the whole approach to cleaning up land far more flexible. The enhanced ability this confers for individually responsive interventions stands as one of the key factors in its wider potential uptake. In this way, for example, fast-response applications can be targeted to bring about a swift initial remediation impact where appropriate, switching over to less engineered or resource-hungry technologies for the long-haul to achieve full and final treatment.

As has been mentioned before, commercial applicability lies at the centre of biotechnology, and process integration has clear economic implications beyond its ability simply to increase the range of achievable remediation. One of the most significant of these is that complex contamination scenarios can be treated more cheaply, by the integrated combination of lower cost techniques. This opens up the way for higher cost individual methods to be used only where absolutely necessary, for example in the case of major contamination events or acute pollution incidents. With limited resources typically available for remediation work, treatment trains offer the possibility of maximising their utilisation by enabling responsible management decisions to be made on the basis of meaningful cost/benefit analysis.

This is an important area for the future, particularly since increased experience of land remediation successes has removed many of the negative perceptions which were previously commonplace over efficiency, speed of treatment and general acceptability. For many years remediation techniques, and bioremediation especially, were seen in a number of countries as just too costly compared with landfill. As changes in waste legislation in several of these regions have driven up the cost of tipping and begun to restrict the amount of biodegradable material entering landfills, the balance has swung the other way, making remediation the cheaper option. There is a certain irony that the very alternative which for so long held back the development of remediation should now provide such a

strong reason for its use. In the future, wider usage of extensive technologies may increase the trend, since they offer the optimum cost/benefit balance, with intensive processes becoming specialised for fast-response or heavy contamination applications. In addition, the 'treatment train' approach, by combining technologies to their maximum efficiency, offers major potential advantages, possibly even permitting applications once thought unfeasible, like diffuse pollution over a large area.

The Suitability of Bioremediation

Bioremediation as a biotechnological intervention for cleaning up the residual effects of previous human activities on a site, typically relies on the inherent abilities and characteristics of indigenous bacteria, fungi or plant species. In the present discussion, the emphasis will concentrate on the contribution made by the first two types of organism. The use of plants, including bioaccumulation, phytoextraction, phytostabilisation and rhizofiltration, all of which are sometimes collectively known as phytoremediation, is examined as part of a separate chapter. Thus, the biological mechanisms underlying the relevant processes are biosorption, demethylation, methylation, metal-organic complexation or chelation, ligand degradation or oxidation. Microbes capable of utilising a variety of carbon sources and degrading a number of typical contaminants, to a greater or lesser extent, are commonly found in soils. By enhancing and optimising conditions for them, they can be encouraged to do what they do naturally, but more swiftly and/or efficiently. This is the basis of the majority of bioremediation and proceeds by means of one of the three following general routes.

Mineralisation, in which the contaminant is taken up by microbe species, used as a food source and metabolised, thereby being removed and destroyed. Incomplete, or staged, decomposition is also possible, resulting in the generation and possible accumulation of intermediate byproducts, which may themselves be further treated by other micro-organisms.

Cometabolism, in which the contaminant is again taken up by microbes but this time is not used as food, being metabolised alongside the organism's food into a less hazardous chemical. Subsequently, this may in turn be *mineralised* by other microbial species.

Immobilisation, which refers to the removal of contaminants, typically metals, by means of adsorption or bioaccumulation by various micro-organism or plant species.

Unsurprisingly, given the expressly biological systems involved, bioremediation is most suited to organic chemicals, but it can also be effective in the treatment of certain inorganic substances and some unexpected ones at that. Metals and radionuclides are good examples of this. Though, obviously, not directly biodegradable themselves, under certain circumstances their speciation can be changed which may ultimately lead to their becoming either more mobile and

Table 5.1 *Potential for bioremediation of selected contaminants*

Readily possible	Possible under certain circumstances	Currently impossible
Acids	Chlorinated solvents	Asbestos
Alcohols	Cyanides	Asphalt
Aldehydes and ketones	Explosives	Bitumen
Ammonia	PCBs	Inorganic acids
Creosote	PAHs	
Chlorophenols	Pesticides, herbicides and fungicides	
Crude oil and petroleum hydrocarbons	Tars	
Glycols	Timber treatments	
Phenols		
Surfactants		

accessible or less so. The net result produced in either case can, under the right conditions, be a very effective functional remediation. A list of typical contaminants suitable for bioremediation would include the likes of crude oil and its derivatives, some varieties of fungicides and herbicides, hydrocarbons, glycols, phenols, surfactants and even explosives.

Developments in bioprocessing continually redefine the definitive catalogue of what may, and may not, be treated and many chemicals once thought 'impossible' are now routinely dealt with biologically. Table 5.1 reflects the current state of the art, though this is clearly subject to change as new approaches are refined.

As a result, it should be obvious that a large number of opportunities exist for which the application of remediating biotechnologies could have potential relevance. Even so, there are a number of factors which affect their use, which will be considered before moving on to discuss practical treatment issues themselves.

Factors Affecting the use of Bioremediation

It is possible to divide these into two broad groups; those which relate to the character of the contamination itself and those which depend on environmental conditions. The former encompass both the chemical nature of the pollutants and the physical state in which they are found in a given incident. Thus, in order for a given substance to be open to bioremediation, clearly it must be both susceptible to, and readily available for, biological decomposition. Generally it must also be dissolved, or at the very least, in contact with soil water and typically of a low–medium toxicity range. The principal environmental factors of significance are temperature, pH and soil type. As was stated previously, bioremediation tends to rely on the natural abilities of indigenous soil organisms and so treatment can occur between 0–50 °C, since these temperatures will be tolerated. However, for greatest efficiency, the ideal range is around 20–30 °C, as

this tends to optimise enzyme activity. In much the same way, a pH of 6.5–7.5 would be seen as optimum, though ranges of 5.0–9.0 may be acceptable, dependent on the individual species involved. Generally speaking, sands and gravels are the most suitable soil types for bioremediation, while heavy clays and those with a high organic content, like peaty soils, are less well indicated. However, this is not an absolute restriction, particularly since developments in bioremediation techniques have removed the one-time industry maxim that clay soils were impossible to treat biologically.

It should be apparent that these are by no means the only aspects which influence the use of remediation biotechnologies. Dependent on the circumstances; nutrient availability, oxygenation and the presence of other inhibitory contaminants can all play an important role in determining the suitability of bioremediation, but these are more specific to the individual application. A number of general questions are relevant for judging the suitability of biological treatment. The areas of relevance are the likes of the site character, whether it is contained or if the groundwater runs off, what contaminants are present, where they are, in what concentrations and whether they are biodegradable. Other typical considerations would be the required remediation targets and how much time is available to achieve them, how much soil requires treatment, what alternative treatment methods are available and at what cost.

Clearly then, there are benefits to the biological approach in terms of sustainability, contaminant removal or destruction and the fact that it is possible to treat large areas with low impact or disturbance. However, it is not without its limitations. For one thing, compared with other technologies, bioremediation is often relatively slower, especially *in situ*, and as has been discussed, it is not equally suitable for all soils. Indeed, soil properties may often be the largest single influence, in practical terms, on the overall functional character of pollution, since they are major factors in modifying the empirical contamination effect. The whole issue may be viewed as hierarchical. The primary influence consists of the contaminants themselves and actual origin of the contamination, which clearly have a major bearing on the overall picture. However, edaphic factors such as the soil type, depth, porosity, texture, moisture content, water-holding capacity, humus content and biological activity may all interact with the primary influences, and/or with each other, to modify the contamination effect, for better or worse. Figure 5.3 is a simplified illustration of this relationship.

Hence, it is not enough simply to consider these elements in isolation; the functional outcome of the same contaminant may vary markedly, dependent on such site-specific differences.

After consideration of the generalised issues of suitability, the decision remains as to which technique is the most appropriate. This is a site-specific issue, for all of the reasons discussed, and must be made on the basis of the edaphic matters mentioned previously, together with proper risk assessment and site surveys. At the end of all these studies and assessments, the site has been investigated by

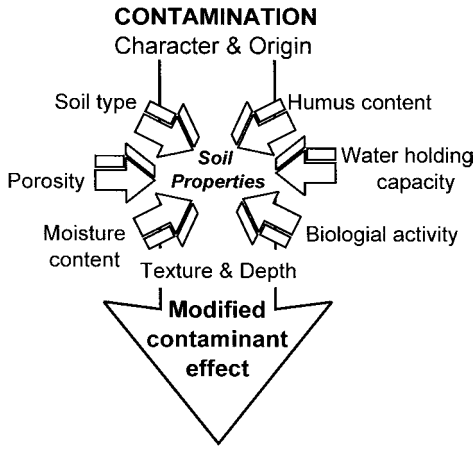

Figure 5.3 *Modification of effect by edaphic factors*

desk top and practical means, empirical data has been obtained, the resident contamination has been characterised and quantified, its extent determined, relevant risk factors identified and risk assessment has been performed. The next stage is the formulation of a remediation action plan, making use of the data obtained to design a response to the contamination which is appropriate, responsible and safe. At this point, having obtained the clearest possible overview, technology selection forms a major part of this process.

When this has been done, and approval has been gained from the relevant statutory, regulatory or licensing bodies, as applicable, the last phase is to implement the remediation work itself.

Biotechnology Selection

Although the primary focus of remediation methods commonly falls on technologies dependent on a relatively high engineering component, there is one purely biological treatment option which can be a very effective means of clean-up. Known variously as 'natural attenuation', 'passive remediation', 'bioattenuation' or 'intrinsic remediation', it is appropriate for sites where the contamination does not currently represent a clear danger to human health or the environment. Though it is not an engineered solution, neither is it a 'do nothing' approach as is sometimes stated, since it is not an exercise in ignoring the problem, but a reasoned decision on the basis of the necessary site investigations, to allow nature to take its course. The approach works with natural cycles and the pre-existing indigenous microbial community to bring about the required treatment. The need for a good initial survey and risk assessment is clear, and typically a comprehensive monitoring programme is established to keep a check on progress.

The effectiveness of natural attenuation has been demonstrated by 20 years or more of research in the USA, which gave rise to the 'Part 503 Rule'. Issued in February 1993, the *Clean Water Act*, specifically the part of it called *Title 40 of the Code of Federal Regulations, Part 503 – The Standards for the Use or Disposal of Sewage Sludge*, which is commonly referred to as the 'Part 503 Rule' or even simply 'Part 503', sets out benchmark limits for the USA.

Typical European regulations follow a precautionary limits model, at times referred to as the 'no net gain or degradation' approach, meaning that there should be no overall accumulation of contaminants in the soil, nor any degradation of the soil quality, compared with original levels.

Part 503 is based on risk assessment of selected key pollutants which pose a threat to humans, other animals or plants, making evaluations of a number of different possible pathways, from a direct, 'single incident' scenario, to a lifetime of possible exposure via bioaccumulation. The standard which is set as a result is based on the lowest concentration which was deemed to present an *acceptable* risk.

In this way, higher heavy metal concentrations and cumulative loading rates are permitted than would be allowed under the Europe model, since the ability of soil to lock them up effectively indefinitely has been demonstrated by extensive research. Accordingly US legislation is based on the principle that even if the background level of a given heavy metal species increases over time, its migration or availability for uptake by plants or animals would be precluded by the combined action of the resident microbes and other general soil characteristics. In many ways this has strong echoes of the soil modification of contaminant effect previously discussed.

The engineered solution

If natural attenuation is not appropriate, then some form of engineered response is required, the selection of which will depend on a number of interlinked factors. Thus, the type and concentration of the contamination, its scale and extent, the level of risk it poses to human health or the environment, the intended eventual site use, the time available for remediation, available space and resources and any site-specific issues, all influence this decision. Many of the key issues have already been discussed and the earlier Figure 5.1 sets out the factors governing technology transition between the *in situ* and *ex situ* techniques.

Essential Features of Biological Treatment Systems

All biotechnology treatments have certain central similarities, irrespective of the specific details of the technique. The majority of applications make use of indigenous, resident microbes, though in some cases the addition of specialised organisms may be warranted. Thus, the functional biology may be described as a process of bioenhancement or bioaugmentation, or occasionally a mixture of both.

Bioenhancement concentrates solely on the existing microfauna, stimulating their activity by the manipulation of local environmental conditions. Bioaugmentation, by contrast, requires the deliberate introduction of selected microbes to bring about the required clean-up. These additions may be unmodified 'wild-type' organisms, a culture selectively acclimatised to the particular conditions to be encountered, or genetically engineered to suit the requirements. Enzyme or other living system extracts may also be used to further facilitate their activity. Some land remediation methods simultaneously bioenhance resident bacteria and bioaugment the process with the addition of fungi to the soil under treatment.

In the final analysis, all biological approaches are expressly designed to optimise the activities of the various micro-organisms (either native to the particular soil or artificially introduced) to bring about the desired remediation. This generally means letting them do what they would naturally do but enhancing their performance to achieve it more rapidly and/or more efficiently. Effectively it is little different from accelerated natural attenuation and typically involves management of aeration, nutrients and soil moisture, by means of their addition, manipulation or monitoring, dependent on circumstance. However simple this appears, the practical implications should not be underestimated and careful understanding of many interrelated factors is essential to achieve this goal. For example, successful aerobic biodegradation requires an oxygen level of at least 2 mg/litre; by contrast, when the major bioremediation mechanism is anaerobic, the presence of any oxygen can be toxic. The presence of certain organic chemicals, heavy metals or cyanides may inhibit biological activity; conversely, under certain circumstances microbial action may itself give rise to undesirable side effects like iron precipitation, or the increased mobilisation of heavy metals within the soil.

In situ techniques

The fundamental basis of *in situ* engineered bioremediation involves introducing oxygen and nutrients to the contaminated area by various methods, all of which ultimately work by modifying conditions within the soil or groundwater. There are three major techniques commonly employed, namely biosparging, bioventing and injection recovery. In many respects, these systems represent extreme versions of a fundamentally unified technology, perhaps best viewed as individual applications of a treatment spectrum as will, hopefully, become clearer from the descriptions of each which follow.

As set out previously, the major benefits of *in situ* methods are their low intrusion, which enables existing buildings and site features to remain undisturbed, their relative speed of commencement and the reduced risk of contamination spread.

Biosparging

Biosparging is a technique used to remediate contamination at, or below, the water table boundary, a generalised diagram of which appears in Figure 5.4. In

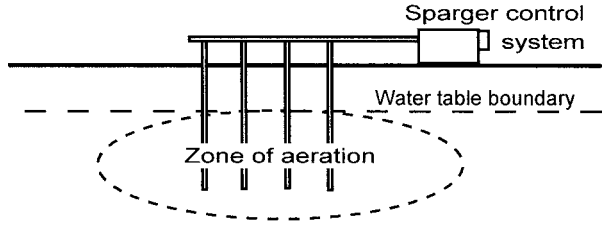

Figure 5.4 *Biosparging*

effect, the process involves superaeration of the groundwater, thereby stimulating accelerated contaminant biodegradation. Though the primary focus of the operation is the saturated zone, the permeability of the overlying soil has a bearing on the process, since increased oxygenation of this stratum inevitably benefits the overall efficiency of remediation.

Air is introduced via pipes sunk down into the contaminated area and forms bubbles in the groundwater. The extra oxygen made available in this way dissolves into the water, also increasing the aeration of the overlying soil, stimulating the activity of resident microbes, which leads to a speeding up of their natural ability to metabolise the polluting substances. The method of delivery can range from relatively simple to the more complicated, dependent on individual requirements. One of the major advantages of this is that the required equipment is fairly standard and readily available, which tends to keep installation costs down. Typically the sparger control system consists of a pressure gauge and relief valve to vent excess air pressure, with associated flow meters and filter systems to clean particulates from the input. More sophisticated versions can also include data loggers, telemetry equipment and remote control systems, to allow for more precise process management. It should be obvious that extensive and comprehensive site investigation, concentrating on site geology and hydrogeology in particular, is absolutely essential before any work starts.

Bioventing

Bioventing is a technique used to remediate contamination above the water table boundary, and again a generalised diagram appears in Figure 5.5. This process also involves superaeration, again with the intention of stimulating accelerated breakdown of the pollutants present, though this time it is taking place within the soil itself, instead of the groundwater. Bioventing is not generally suitable for remediating sites with a water table within one metre of the surface, nor for heavy or waterlogged soils, since air flow is compromised under these conditions.

Air is introduced from a compressor pump, via a central pipe, or set of pipes, dependent on the size of the area to be treated, down into the region of contamination. The extra oxygen availability thus achieved, as in the previous approach described, stimulates the resident microbes, which then treat

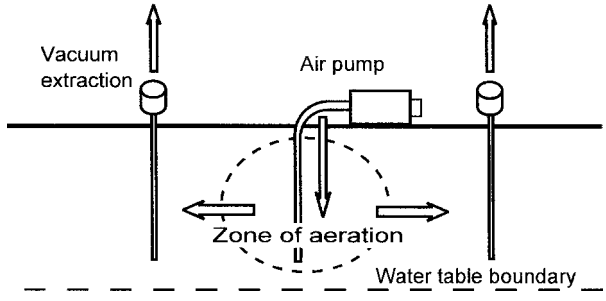

Figure 5.5 *Bioventing*

the polluting substances. The air flow through the soil is further driven by vacuum extractors peripheral to the treatment zone, which increases the dissolved oxygen levels of the soil water and thus facilitates uptake by the native micro-organisms. Volatile compounds, which are either present as part of the original contamination, or generated as byproducts of the biological treatment, are often mobilised during processing and thus more easily extracted. However, in many practical applications, the air extraction rate is adjusted to maximise decomposition underground, thus reducing a separate requirement for surface treatment of volatile compounds.

As with the biosparger, control devices typically regulate the pressure, filters clean particles from the intake and the flow rate is monitored in operation, with data loggers and telemetry systems again featuring in the more complex applications.

Unsurprisingly, bioventing also requires extensive and comprehensive site investigation before commencement, not least because the proper positioning of the necessary system of pipework is essential to the proper functioning of this technique.

Injection recovery

The injection and recovery method, for which a generalised diagram appears in Figure 5.6, makes use of the movement of groundwater through the zone of contamination to assist the remediation process. Although, as mentioned in the introductory comments, this approach shares many functional similarities with the preceding technologies, it is essentially more sophisticated and refined, with the biological treatment being effectively divided into two complementary stages. Thus, what may be considered a ‘virtual’ bioreactor is established within the soil matrix, with the actual clean-up activity taking place both within the groundwater and also externally to it.

The major characteristic of this technique is the two-well system sunk into the ground, the ‘injection well’ and the ‘recovery well’, the former being located

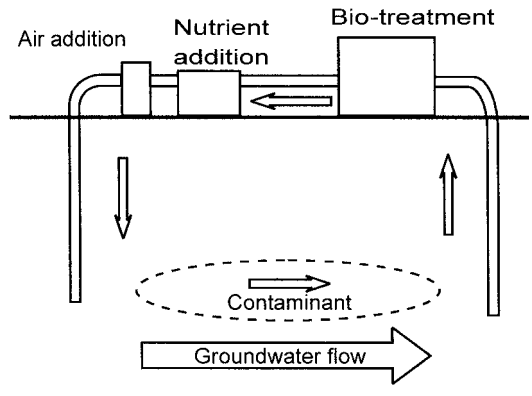

Figure 5.6 *Injection recovery*

'upstream' of the latter. Nutrients and air are forced down the injection well, and as they flow through the contamination, they stimulate the growth and activity of the indigenous micro-organisms, which begin the process of remediation.

Groundwater, now rich in contaminant, microbes, microbial metabolites and contaminant breakdown products is extracted via the 'recovery well' from beyond the contaminated zone. It then undergoes additional biological treatment above ground in an associated bioreactor vessel, frequently where it is subjected to highly aerobic conditions, before being reinjected, having been further replenished with air and nutrients. This cycle may be repeated many times in the course of treatment. Process control is achieved by having separated out the aeration, nutrient addition and biotreatment phases into isolated near-episodic events and the facility for direct analysis of the abstracted water enables treatment progress to be monitored with much greater certainty. As a consequence, the injection recovery method neatly overcomes many of the traditional criticisms of *in situ* treatment techniques, particularly in respect of difficulties in ensuring true optimisation of conditions and determining the end-point.

Of course, this technique does not avoid the necessity for thorough site investigation and geological surveys, since it is clearly imperative that the particulars of the subterranean water flow, soil depth and underlying geology are known in considerable detail.

Site monitoring for biotechnological applications

Environmental monitoring is well established as a separate science in its own right and many notable books have been written to describe the various approaches and techniques relevant to its many practical applications. It is then, clearly, beyond the scope of this work to reiterate these discussions and the reader is recommended to examine such publications at first hand should detailed information be required.

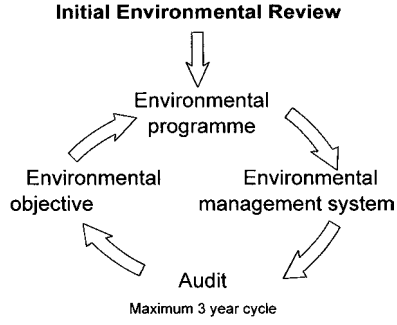

Figure 5.7 *Illustrative long-term monitoring scheme*

However, it is worth noting that for some sites it may be necessary to continue monitoring into the future. Under these circumstances, a comprehensive environmental management and audit scheme can be put in place to monitor environmental effects of such operations and Figure 5.7 shows a suitable illustrative outline. The results would then, of course, feed back into the decision-making process and ultimately help to shape the ongoing environmental management regime of the site.

Ex situ techniques

Again, there are three principal approaches in common use, namely land farming, soil banking and soil slurry bioreactors. Though inevitably there are distinct similarities between all applications of bioremediation, for obvious reasons of fundamental biology, these techniques are generally more distinct and separate.

The major benefits of *ex situ* methods are the greater ease of process optimisation and control, relatively shorter treatment time and the increased potential for the safe introduction of specialised organisms, if and as required. However, the increased transport costs, additional land requirement and higher levels of engineering often combine to make these technologies more costly options.

Land farming

This technique is effectively accelerated natural attenuation, taking place offsite, within constructed earthwork banking to provide what is essentially a low-tech bioreactor. The pretreatment stage involves the soil being excavated from site, screened for rocks, rubble and any other oversize inclusions before typically being stored prior to the commencement of actual remediation, either at the original location or on arrival at the treatment site.

The processing itself takes place in lined earthworks isolated from the surroundings by an impermeable clay or high density polyethylene (HDPE) liner, as shown diagrammatically in Figure 5.8, and typically relies on the activities

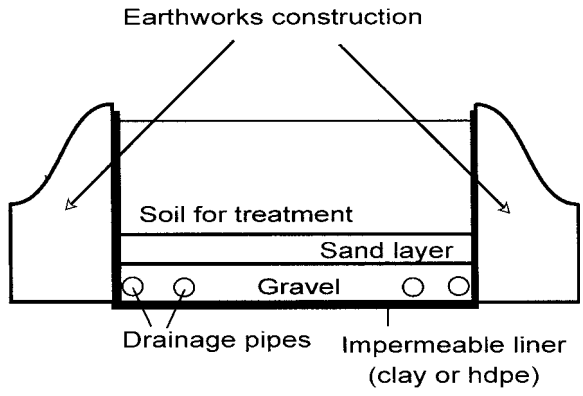

Figure 5.8 *Schematic diagram of land farming*

of indigenous micro-organisms to bring about the remediation, though specialist bacteria or fungi can be added if required. The soil to be treated is laid on a sand layer, which itself stands on a gravel bed, through which a series of drainage pipes have been laid. An impermeable clay or polymer lining isolates the whole system from direct contact with the underlying ground. Water and nutrients are added to stimulate biological activity and soil aeration is maintained by means of turning or ploughing.

The inherent simplicity of the process, however, makes its effectiveness highly dependent on soil characteristics and climatic conditions. For example, heavy clay soils, make attaining adequate oxygenation difficult and uniform nutrient distribution is almost impossible to achieve. In colder climes, it may be necessary to cover the soil to overcome the worst effects of the weather.

Throughout the treatment itself, a process of sampling and monitoring helps to assess progress and compliance with required standards and, at completion, the treated soil can be removed either for return to original site or use elsewhere.

Soil banking

In some respects, soil banking is an inverted version of the previous system, ranging from a long row of soil at its simplest, to a more engineered approach, with aeration pipes, a drainage layer, impermeable liner and a reservoir to collect leachate.

Just as with the previous approach, soil is excavated and screened, often also being stored prior to treatment. As the name suggests, the soil to be processed is formed into banks, sometimes with the addition of filler material like chaff, wood chips or shredded organic matter, if the character of the contaminated soil requires it to improve the overall texture, ease of aeration, water-holding capacity or organic matter content. This technique is sometimes termed ‘soil composting’

because of the similarity it has with the windrow method of treatment for biowaste material, which is described in Chapter 8. It is not a true example of the compost process, though there are many functional parallels between these procedures and the same windrow turning equipment may be used in either. Often these rows are covered, either with straw or synthetic blanketing materials, to conserve heat and reduce wash-out. Accordingly, this method is generally better suited to colder and wetter climates and is typically faster than land farming. Indigenous micro-organisms are again the principal agents of remediation, though specialised bacterial or fungal cultures can be introduced as required, and nutrients added to optimise and enhance their activities.

To further boost the speed and efficiency of this treatment approach, particularly when space is limited, a more sophisticated version, often termed 'engineered biopiling', is sometimes used to ensure greater process control. Leachate is collected in a reservoir and recirculated through the pile to keep the soil moist and return the microbes it contains and a series of pipes within the pile or the underlying drainage layer forces air through the biopile itself. The increased air flow also permits VOCs to be managed more efficiently and having the whole system above an impermeable geotextile liner prevents leachate migration to the underlying ground.

In both versions of soil banking, a regime of sampling and monitoring is established which again aids process assessment and control. After treatment is concluded, the soil may be returned to the original site for use, or taken elsewhere.

Both land farming and soil banking are relatively unsophisticated approaches, effectively utilising the mechanisms of natural attenuation to bring about the necessary clean-up, though enhancing and accelerating the process, having first isolated, concentrated and contained the material to be treated. The final commonly encountered technology to be described in this section is a more engineered approach, which works by increasing the levels of water, nutrients and dissolved oxygen available to the microbes.

Soil slurry reactor

In most respects, this system shares essentially similar operating principles to the activated sludge system described in the next chapter, which is used in treating effluents. Figure 5.9 shows a schematic representation of this method.

After excavation, the soil is introduced into a mixing tank, where a slurry is produced by combining it with water. Nutrients are then added to stimulate microbial growth. The suspension formed is transferred to a linked series of well-aerated slurry reactors, and micro-organisms within them progressively treat the contaminants. Clarifiers and presses thicken the treated slurry and dewater it, the recovered liquid component being recirculated to the mixing tank to act as the wetting agent for the next incoming batch of soil, while the separated solids are removed for further drying followed by reuse or disposal.

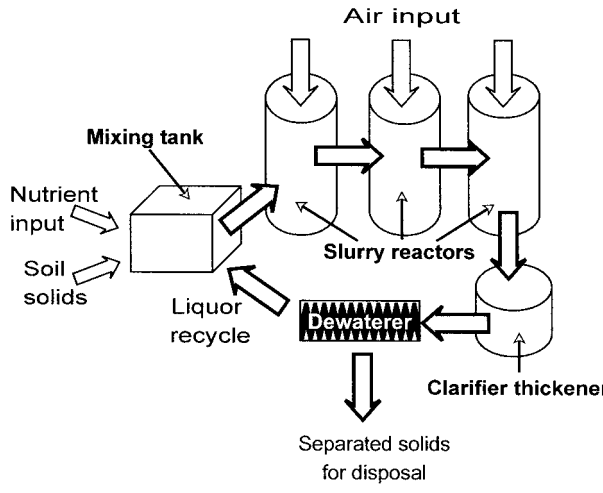

Figure 5.9 *Schematic soil slurry bioreactor system*

Process selection and integration

However, when complex mixtures of compounds are required to be treated, combining a series of different individual process stages within a series of interlinked bioreactors, may often be a more appropriate and effective response. Dependent on the specific type of contaminants, this may necessitate a sequence of both aerobic and anaerobic procedures, or even one which combines biological and chemical steps to achieve the optimum remediation system. Under such circumstances, clearly each bioreactor features conditions designed to optimise specific biological processes and degrade particular contaminants.

It should be clear from the preceding discussions that the actual process of bioremediation employed will depend on a number of factors, amongst others relating to the site itself, the local area, economic instruments, reasons for remediation and the benefits and limitations of the actual technologies. Hence, it should not be difficult to see that for any given contamination event, there may be more than one possible individual approach and, indeed, as described earlier, the potential will often exist for using integrated combinations of technologies to maximise the effectiveness of the overall response. In this way, though dependent on many external variables, a mix and match assemblage of techniques may represent the individual best practicable environmental option (BEPO). The merging of an *ex situ* treatment, like, for example, soil washing via a slurry reactor, to offer an intensive and immediate lessening of pollution effect, with a slower *in situ* process to polish the site to a final level, has much to recommend it, both environmentally and commercially. Accordingly, it seems reasonable to conclude that the prevalence and relative importance of such approaches will be likely to grow over the coming years.

Use of remediation techniques

As was stated earlier, there are several remediation techniques available, of which bioremediation is just one and, for the most part, regional variables define which approach will tend to be the more commonly used for any given country. In the United Kingdom, guideline figures are available from BioWise, the government-established body charged with promoting the use of biotechnology, formerly 'Biotechnology Means Business' (BMB). These indicated that of the seven most commonly available methods in 1997, containment and encapsulation accounted for 46% of remediation activity in the UK, excavation for disposal made up 28%, with bioremediation in third place at 12%. The remaining 14% was achieved by vacuum extraction (7%), chemical treatment (4%), solvent washing (2%) and finally, at 1%, incineration as shown in Figure 5.10.

Though this may be of limited relevance in universal terms, since, as has been pointed out throughout, the situation in one country does not necessarily bear any resemblance to that in another, in many ways, it does serve as a useful illustration of the link between economics and the uptake of environmental biotechnology. Over the same period, the costs for remediation were as shown in Table 5.2.

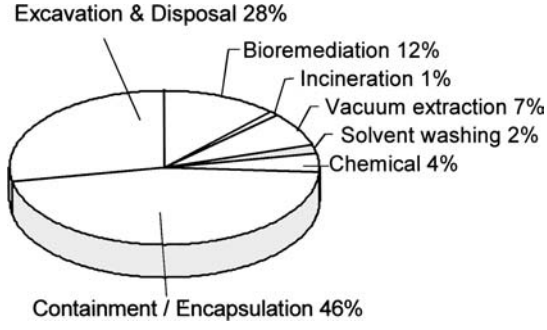

Figure 5.10 *Pie chart of remediation technologies use in the UK (1997)*

Table 5.2 *A cost comparison of selected technologies*

Technology	Typical cost (£/m^3)
Bioremediation	10–80
Chemical	10–100
Encapsulation	20–180
Excavation/Disposal	30–75
Incineration	100–400
Soil washing	15–40

Source: Biotechnology Means Business 1996/7 figures.

Closing Remarks

While there is considerable overlap in technology costs, the economic element is very largely identifiable in the general trends of use and, of course, biotechnology is not applicable to all forms of pollution. The situation for bioremediation at least seems set to benefit further as (in the UK) landfill tax, the escalating costs of special waste disposal, and the extra demands of the EU Landfill Directive, which is discussed in some detail in Chapter 8, have all combined to make biological treatment an increasingly cost-effective option. The UK is by no means unique in this respect. Similar changes have helped to make clean-up biotechnologies more competitive elsewhere in the world also and it seems this trend is likely to continue into the foreseeable future.

References

BioWise, UK Department of Trade and Industry (2001) *Contaminated Land Remediation: A Review of Biological Technology*, Crown copyright.

Case Study 5.1 Oil Bioremediation (Texas, USA)

Sites contaminated by petroleum hydrocarbons are often particularly well suited to bioremediation and there are many examples across the globe of the successful use of biotechnology in such clean-up operations.

One particularly successful example, closely monitored by the Texas Parks and Wildlife Department and the Texas Railroad Commission, involved the treatment of petroleum-contaminated soil at a site within Tyler State Park, Texas, located close to a large recreational lake. Historic oil exploration, pumping and gathering operations had resulted in significant petroleum hydrocarbon contamination and the crude oil storage tanks, which had been removed before the project began, had left behind actual puddles of oil, in some places, though the contamination was generally of shallow character, only extending to around 0.3–0.5 metres in depth.

ETTL engineers, who had been engaged to do the clean-up, employed a process using indigenous soil microbes harvested from the site, which were then artificially multiplied in the laboratory before being reintroduced in a solution that also contained nutrients and biosurfactants.

The *in situ* treatment process used was a land-farming method, with the soil being deeply ploughed before being sprayed with the microbial solution. The soil was further tilled at fortnightly intervals to aerate it and keep its structure open, and the hydrocarbon concentration, pH, soil temperature, and moisture levels were monitored throughout. This allowed additional water, nutrients and pH buffers to be added as required, to maximise the efficiency of the bioremediation system. The final outcome exceeded the minimum legal requirements by a large margin and at a cost significantly lower than traditional methods and slightly under that originally anticipated.

Case Study 5.2 Annelid Bioreactors (United Kingdom)

The principle of providing optimised conditions for microbial action is well established, but actually achieving this is one of the biggest practical difficulties for the bioremediation of contaminated land. This is a significant potential problem for *in situ* systems, because the soil is treated lying in its natural place, thus making control of the microbes' environment difficult to achieve. In particular, ensuring maximum contact between the microbes and the contaminated soil, the provision of adequate aeration and the maintenance of suitable moisture content can be very difficult.

An approach proposed, and currently being patented, by Taeus Biotech involves using species of earthworm to act as discrete, self-propelled bioreactors, effectively bringing the benefits of *ex situ* remediation to an *in situ* context, thereby overcoming many of the latter's shortcomings at the same time.

Once the nature of the contamination has been established, a suitable culture of microbes with the necessary competence to degrade it can be produced, typically drawing on micro-organisms indigenous to the site, training them as required. Appropriate earthworm varieties are kept in growing units, which contain a soil medium which has been heavily inoculated with the microbial culture. The natural burrowing and feeding activities of the worms ensure that they pick up large numbers of microbes, which then remain resident in their gut.

The prepared earthworms are then introduced to the contaminated site and assist in its remediation in two main ways. Firstly, as they move around, the contaminated soil they ingest is brought into direct contact with the cultured micro-organisms, while mucus-secreting glands within their gut maintain adequate internal moisture. Secondly, many of the competent microbes are carried out in the soil, as it passes through the worms, effectively becoming an *in situ* site inoculant to multiply and continue the clean-up process, aided by the improved aeration provided by the earthworm burrows.

This technique is still in the first stages of development and it is, clearly, limited to certain kinds of contamination, present in relatively low concentrations. However, for such instances, particularly when the pollution is widespread throughout a site, the early indications are that it could be a cost-effective means of low intervention bioremediation.

6
Aerobes and Effluents

The easiest thing that a sewage works is required to treat is sewage; a large number of industrial or commercial activities produce wastewaters or effluents which contain biodegradable contaminants and typically these are discharged to sewers. The character of these effluents varies greatly, dependent on the nature of the specific industry involved, both in terms of the likely BOD loading of any organic components and the type of additional contaminants which may also be present. Accordingly, the chemical industry may offer wastewaters with high COD and rich in various toxic compounds, while tannery water provides high BOD with a chromium component and the textile sector is another high BOD effluent producer, with the addition of surfactants, pesticides and dyes. Table 6.1 shows illustrative examples of typical effluent components for various industry sectors.

Table 6.2 shows the illustrative effluent BOD by industry sector, from which it should be apparent that the biodegradable component of any given wastewater is itself highly variable, both in terms of typical values between industries, but also in overall range. Thus, while paper pulping may present an effluent with a BOD of 25 000 g/m^3, sewage returns the lowest of the BODs quoted, clearly underlining the point of this chapter's opening statement.

As might reasonably be expected from the foregoing discussion, the direct human biological contribution to wastewater loading is relatively light. A 65 kg person produces something in the region of 0.1–0.5 kg of faeces per day on a wet weight basis, or between 30–60 g of dry solids. The same person also produces around 1–1.5 kg of urine per day, with a total mass of dry solids amounting to some 60–80 g. Of course, the actual effluent arriving at a sewage works for treatment contains the nitrogen, phosphorus and other components originally excreted in the urine or faeces, but in a higher dilution due to flushing water and, often, storm drainage also. Local conditions, climate, details of the sewer system and water availability are, clearly, all potential factors affecting this, though a 49:1 ratio of water to solids is fairly typical for developed nations.

Sewage Treatment

Looking at sewage works in the strictly literal term, the aims of treatment can be summarised as the reduction of the total biodegradable material present, the

Table 6.1 *Illustrative examples of typical effluent components by industry sector*

Industry sector	Typical effluent component
Chemical industry	High COD, toxic compounds
Distillery	High BOD
Engineering	Oils, metals
Food processing	Fats, starches, high BOD
Paper pulping	Very high BOD, bleaches
Tanning	High BOD, chromium
Textile manufacture	High BOD, surfactants, pesticides, dyestuff
Timber	Preservatives, fungicides

Table 6.2 *Illustrative examples of typical effluent BOD by industry sector*

Industry sector	Typical BOD
Abattoir	2 600
Brewery	550
Distillery	7 000
Landfill leachate	20 000
Paper pulping	25 000
Petroleum refinery	850
Sewage	350
Tannery	2 300

All values in g/m^3.

removal of any co-existing toxic substances and the removal and/or destruction of pathogens. It is beyond the scope of this book to examine the general, non-biological processes of sewage treatment in great detail, but for the sake of establishing the broader context in which the relevant biotechnology functions, a short description of the main key events follows. It is not, nor is it intended to be, a comprehensive examination of the physical processes involved and the reader is urged to consult relevant texts if this information is authoritatively required.

The typical sewage treatment sequence normally begins with preliminary screening, with mechanical grids to exclude large material which has been carried along with the flow. Paper, rags and the like are shredded by a series of rotating blades known as comminutors and any grit is removed to protect the pumps and ensure free movement of the water through the plant. Primary treatment involves the removal of fine solids by means of settlement and sedimentation, the aim being to remove as much of the suspended organic solid content as possible from the water itself and up to a 50% reduction in solid loading is commonly achieved. At various times, and in many parts of the world, discharge of primary effluent direct to the sea has been permissible, but increasing environmental legislation means that this

has now become an increasingly rare option. Throughout the whole procedure of sewage treatment, the effective reduction of nitrogen and phosphorus levels is a major concern, since these nutrients may, in high concentration, lead to eutrophication of the waterways. Primary stages have a removal efficiency of between 5–15% in respect of these nutrients, but greater reductions are typically required to meet environmental standards for discharge, thus necessitating the supernatant effluent produced passing to a secondary treatment phase. This contains the main biological aspect of the regime and involves the two essentially linked steps of initial bioprocessing and the subsequent removal of solids resulting from this enhanced biotic activity. Oxidation is the fundamental basis of biological sewage treatment and it is most commonly achieved in one of three systems, namely the percolating filter, activated sludge reactor or, in the warmer regions of the globe, stabilisation ponds. The operational details of the processing differ between these three methods and will be described in more detail later in this section, though the fundamental underlying principle is effectively the same. Aerobic bacteria are encouraged, thriving in the optimised conditions provided, leading to the BOD, nitrogen and ammonia levels within the effluent being significantly reduced. Secondary settlement in large tanks allows the fine floc particles, principally composed of excess microbial biomass, to be removed from the increasingly cleaned water. The effluent offtake from the biological oxidation phase flows slowly upwards through the sedimentation vessels at a rate of no more than 1–2 metres per hour, allowing residual suspended solids to settle out as a sludge. The secondary treatment stage routinely achieves nutrient reductions of between 30–50%.

In some cases, tertiary treatment is required as an advanced final polishing stage to remove trace organics or to disinfect effluent. This is dictated by watercourse requirements, chiefly when the receiving waters are either unable to dilute the secondary effluent sufficiently to achieve the target quality, or are themselves particularly sensitive to some component aspect of the unmodified influx. Tertiary treatment can add significantly to the cost of sewage management, not least because it may involve the use of further sedimentation lagoons or additional processes like filtration, microfiltration, reverse osmosis and the chemical precipitation of specific substances. It seems likely that the ever more stringent discharge standards imposed on waterways will make this increasingly commonplace, particularly if today's concerns over nitrate sensitivity and endocrine disrupters continue to rise in the future.

Process Issues

At the end of the process, the water itself may be suitable for release but, commonly, there can be difficulty in finding suitable outlets for the concentrated sewage sludge produced. Spreading this to land has been one solution which has been successfully applied in some areas, as a useful fertiliser substitute on agricultural or amenity land. Anaerobic digestion, which is described more fully

in the context of waste management in Chapter 8, has also been used as a means of sludge treatment. The use of this biotechnology has brought important benefits to the energy balance of many sewage treatment works, since sludge is readily biodegradable under this regime and generates sizeable quantities of methane gas, which can be burnt to provide onsite electricity.

At the same time, water resources are coming under increasing pressure, either through natural climatic scarcity in many of the hotter countries of the world, or through increasing industrialisation and consumer demand, or both. This clearly makes the efficient recycling of water from municipal works of considerable importance to both business and domestic users.

Though in many respects the technology base of treatment has moved on, the underlying microbiology has remained fundamentally unchanged and this has major implications, in this context. In essence, the biological players and processes involved are little modified from what would be found in nature in any aquatic system which had become effectively overloaded with biodegradable material. In this way, a microcosmic ecological succession is established, with each organism, or group, in turn providing separate, but integrated, steps within the overall treatment process. Hence, heterotrophic bacteria metabolise the organic inclusions within the wastewater; carbon dioxide, ammonia and water being the main byproducts of this activity. Inevitably, increased demand leads to an operational decrease in dissolved oxygen availability, which would lead to the establishment of functionally anaerobic conditions in the absence of external artificial aeration, hence the design of typical secondary treatments. Ciliate protozoans feed on the bacterial biomass produced in this way and nitrifying microbes convert ammonia first to nitrites and thence to nitrates, which form the nitrogen source for algal growth. Though the role of algae in specifically engineered, plant-based monoculture systems set up to reduce the nitrogen component of wastewaters is discussed more fully in the next chapter, it is interesting to note, in passing, their relevance to a 'traditional' effluent treatment system.

One of the inevitable consequences of the functional ecosystem basis underlying sewage treatment plants is their relative inability to cope with toxic chemicals which may often feature in certain kinds of industrial wastewaters. In particular, metabolic poisons, xenobiotics and bactericidal disinfectants may arrive as components of incoming effluents and can prove of considerable challenge to the resident microbes, if arriving in sufficient concentration. This is a fact often borne out in practice. In 2001, considerable disruption was reported as a result of large quantities of agricultural disinfectant entering certain sewage works as a consequence of the UK's foot and mouth disease outbreak. A number of potential consequences arise from such events. The most obvious is that they kill off all or part of the biological systems in the treatment facility. However, dependent on the nature of the substances, in microbially sublethal concentrations, they may either become chemically bound to either the biomass or the substrate, or be subject to incomplete biodegradation. The effective outcome of this is

that the degree of contaminant removal achievable becomes uncertain and less easily controlled. Partial mineralisation of toxic substances is a particular concern, often leading to the accumulation of intermediate metabolites in the treated wastewater, which may represent the production of a greater biological threat. The incomplete metabolism of these chemicals under aerobic conditions typically results in oxidised intermediary forms which, though less intrinsically toxic than their parent molecules, are often more mobile within the environment. In addition, when the treatment efficiency is subject to monitoring, as intermediate metabolites, these substances may not be picked up by standard analytical techniques, which may result in an unfairly high measure of pollutant removal being obtained.

Moreover, the extension of sewage treatment facilities to ameliorate trade effluents also has implications for the management of true sewage sludge. It is not economically viable to develop processing regimes which do not lead to the concentration of toxic contaminants within the derived sludge. This was shown to be a particular problem for plants using the activated sludge process, which relies on a high aeration rate for pollutant removal, which proceeds by making use of biotransformation, air stripping and adsorption onto the biomass. Adsorption of toxic inorganic substances like heavy metals, or structurally complex organic ones, onto the resident biomass, poses a problem when the microbial excess is removed from the bioreactor, particularly since dewatering activities applied to the extracted sludge can, in addition, catalyse a variety of chemical transformations. Accordingly, sewage sludge disposal will always require careful consideration if the significant levels of these chemicals are not subsequently to cause environmental pollution themselves.

Land Spread

The previous chapter discussed the inherent abilities of certain kinds of soil microbes to remediate a wide range of contaminants, either in an unmodified form, or benefiting from some form of external intervention like optimisation, enhancement or bioaugmentation. Unsurprisingly, some approaches to sewage treatment over the years have sought to make use of this large intrinsic capacity as an unengineered, low-cost response to the management of domestic wastewaters. Thus, treatment by land spread may be defined as the controlled application of sewage to the ground to bring about the required level of processing through the physico-chemical and biological mechanisms within the soil matrix. In most applications of this kind, green plants also play a significant role in the overall treatment process and their contribution to the wider scope of pollutant removal is discussed more fully in the next chapter.

Although it was originally simply intended as a disposal option, in a classic case of moving a problem from one place to another, the modern emphasis is firmly on environmental protection and, ideally, the recycling of the nutrient

component. The viability of land treatment depends, however, on the prevention of groundwater quality degradation being afforded a high priority. In the early days of centralised sewage treatment, the effluent was discharged onto land and permitted to flow away, becoming treated over time by the natural microbial inhabitants of the soil. This gave rise to the term a 'sewage farm' which persists today, despite many changes in the intervening years. Clearly, these systems are far less energy intensive than the highly engineered facilities common in areas of greater developed urbanisation.

The most common forms of effluent to be treated by land spread, or the related soil injection approach, are agricultural slurries. According to the European Commission's Directorate General for Environment, farm wastes account for more than 90% of the waste spread on land in Europe and this is predominantly animal manure, while wastes from the food and beverage production industry form the next most important category (European Union 2001a). Removal of the constituent nutrients by soil treatment can be very effective, with major reductions being routinely achieved for suspended solids and BOD. Nitrogen removal generally averages around 50% under normal conditions, though this can be significantly increased if specific denitrifying procedures are employed, while a reduction in excess of 75% may be expected for phosphorus. Leaving aside the contribution of plants by nutrient assimilation, which features in the next chapter, the primary mechanisms for pollution abatement are physical filtration, chemical precipitation and microbiological metabolism. The latter forms the focus of this discussion, though it should be clearly understood that the underlying principles discussed in the preceding chapter remain relevant in this context also and will not therefore be lengthily reiterated here.

The activity is typically concentrated in the upper few centimetres of soil, where the individual numbers of indigenous bacteria and other micro-organisms are huge and the microbial biodiversity is also enormous. This natural species variety within the resident community is fundamental to the soil's ability to biodegrade a wide range of the components in the wastewaters applied to it. However, it must be remembered that the addition of exogenous organic material is itself a potential selective pressure which shapes the subsequent microbial complement, often bringing about significant alterations as a result. The introduction of biodegradable matter has an effect on the heterotrophic micro-organism population in both qualitative and quantitative terms, since initially there will tend to be a characteristic dying off of sensitive species. However, the additional nutrients made available, stimulate growth in those organisms competent to utilise them and, though between influxes, the numerical population will again reduce to a level which can be supported by the food sources naturally available in the environment, over time these microbes will come to dominate the community. In this way, the land spreading of wastewater represents a selective pressure, the ultimate effect of which can be to reduce local species diversity. Soil experiments have shown that, *in extremis*, this can produce a ten-fold drop in fungal species

and that *Pseudomonas* species become predominant in the bacterial population (Hardman, McEldowney and Waite 1994).

With so high a resident microbial biomass, unsurprisingly the availability of oxygen within the soil is a critical factor in the efficiency of treatment, affecting both the rate of degradation and the nature of the end-products thus derived. Oxygen availability is a function of soil porosity and oxygen diffusion can consequently be a rate-limiting step under certain circumstances. In general, soils which permit the fast influx of wastewater are also ideal for oxygen transfer, leading to the establishment of highly aerobic conditions, which in turn allow rapid biodegradation to fully oxidised final products. In land that has vegetation cover, even if its presence is incidental to the treatment process, most of the activity takes place within the root zone. Some plants have the ability to pass oxygen derived during photosynthesis directly into this region of the substrate. This capacity to behave as a biological aeration pump is most widely known in relation to certain aquatic macrophytes, notably *Phragmites* reeds, but a similar mechanism appears to function in terrestrial systems also. In this respect, the plants themselves are not directly bioremediating the input effluent, but acting to bioenhance conditions for the microbes which do bring about the desired treatment.

Septic tank

In many respects, the commonest rural solution to sewage treatment beyond the reach of sewerage, namely the septic tank, makes use of an intermediate form of land treatment. In the so-called cesspit, a sealed underground tank, collects and stores all the sewage arising from the household. At regular intervals, often around once a month dependent on the capacity, it requires emptying and tankering away, typically for spreading onto, or injection into, agricultural land. By contrast, a septic tank is a less passive system, settling and partially digesting the input sewage, although even with a properly sized and well-managed regime the effluent produced still contains about 70% of the original nutrient input. In most designs, this is mitigated by the slow discharge of the liquid via an offtake pipe into a ground soakaway, introducing the residual contaminants into the soil, where natural treatment processes can continue the amelioration of the polluting constituents. There are various types of septic systems in use around the world, though the most common, illustrated in Figure 6.1, is made up of an underground tank, which is linked to some form of *in situ* soil treatment system, which usually consists of a land drainage of some kind.

Since a system that is poorly designed, badly installed, poorly managed or improperly sited can cause a wide range of environmental problems, most especially the pollution of both surface and groundwaters, their use requires great care. One of the most obvious considerations in this respect is the target soil's ability to accept the effluent adequately for treatment to be a realistic possibility

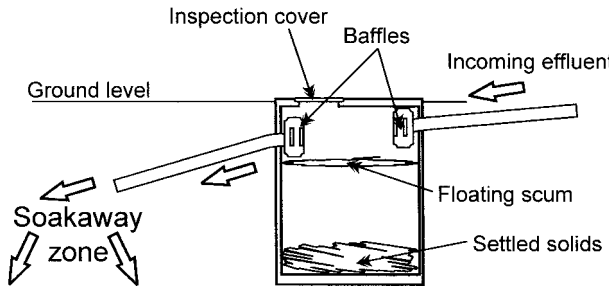

Figure 6.1 *Diagrammatic septic tank*

and hence the percolation and hydraulic conductivity of the ground are important factors in the design and long-term success of this method.

Under proper operation, the untreated sewage flows into the septic tank, where the solids separate from the liquids. Surfactants and any fat components tend to float to the top, where they form a scum, while the faecal residues remaining after bacterial action sink to the bottom of the tank, to form a sludge. The biodegradation of the organic effluent in these systems is often only partially complete and so there tends to be a steady accumulation of sediment within the tank, necessitating its eventual emptying. This settling effect produces a liquid phase which is permitted to flow out of the tank, along an overflow pipe situated towards the top of the vessel and is discharged to the soil as previously described. Internal baffles inside the tank are designed to retain the floating scum layer and prevent undegraded faeces from leaving the system prematurely. If these biosolids were permitted to wash out into the soil its ability to treat the septic-tank effluent can readily become compromised, leading to a reduction in the overall system efficiency.

The drainage arrangements associated with a septic tank system are, arguably, perhaps the most important part of this whole approach to sewage treatment and may be considered as effectively forming an underground microbiological processing plant. Clearly, it is of vital importance that the soil on any given site must be suitable for the drainage to function reliably. The only way to be certain is, of course, by means of a percolation test, though as a general rule, clay soils are unsuited to this purpose. In circumstances of defined clay strata, particularly when they exist close to the surface, it is highly unlikely that straightforward drainage arrangements will prove satisfactory. Even in the absence of a high clay content, soils which are either too fine or very coarse can also reduce the effectiveness of this phase of the treatment system. The former can be a problem because, like clay, it also resists effluent infiltration, the latter because it permits it too quickly and thus retention time becomes inadequate for the level of treatment needed.

A further consideration which must be addressed in this respect is the position of the water table, which may cause problems for the drainage system if it

lies within half a metre of the surface. Consequently in areas where this is a permanent or even seasonal feature, the drains may be established much higher than would be typical, frequently in close proximity to the soil surface. This brings its own inevitable set of concerns, not least amongst them being that there can be a very real possibility of the relatively untreated effluent breaking through to above ground.

One solution to this potential problem that has been used with some success is the sewage treatment mound. Formed using clean sand or small gravel, the mound elevates the system so that it sits a metre or so above the level of the seasonal highest water table. The construction of the mound needs to receive careful consideration to produce a design which suits the local conditions, while also guaranteeing an even distribution of the septic tank effluent throughout the mound. Typically, these systems are intermittently fed by a pump from a collection point and the rate at which the liquid off-take flows through the soil is a critical factor in the correct sizing of the drainage mound. In the final analysis, the sizing of all septic tank systems, irrespective of the details of its specific design, depends on the amount of sewage produced, the type and porosity of soil at the site and the rate at which water flows through it. Proper dimensional design and throughput calculations are of great importance, since the efficacy of septic systems is readily reduced when the set-up is overloaded.

Most modern installations use premanufactured tanks, typically made of stable polymer and formed in a spherical shape with a short shaft like the neck of a bottle forming a ground level inspection point. They often have a series of internal baffles moulded within them to facilitate the flow of liquids and retention of solids and surface scum, together with the appropriate pipework inlets, outlets and gas vents. This type of tank has become increasingly popular since they are readily available, easier to site and can be operational much faster than the older concrete designs.

The most common versions of these consisted of two rectangular chambers which were originally built out of brick or stone until the advent of techniques to cast concrete *in situ*. Sewage digestion was incompletely divided into two stages, with gas venting from the primary chamber and secondary also, in better designed systems. These were sometimes associated with an alternative soil-dosing phase, known as seepage pits and soakaways, in which the part-treated effluent arising from the septic tank is discharged into a deep chamber, open to, and contiguous with, the soil at its sides and base. This permitted the free translocation of liquid from the seepage pit into the surrounding soil, the whole of the surrounding ground becoming, in effect, a huge soakaway, allowing dilution and dispersal of the effluent and its concomitant biotreatment within the body of the soil. In practice, provided the character of the ground is truly suitable for this approach, effluent infiltration and remediation can be very effective. However, if the soil porosity precludes adequate percolation, the potential problems are obvious.

Limits to land application

There are, then, limits to the potential for harnessing the processes of natural attenuation for effluent treatment. While centuries of use across the world testify to the efficacy of the approach for human sewage and animal manures, its application to other effluents is less well indicated and the only truly 'industrial' wastewaters routinely applied to the land in any significant proportion tend to be those arising from food and beverage production. This industry is a consumer of water on a major scale. Dairy production uses between 2–6 m^3 of water per 1 m^3 of milk arriving at the plant, the manufacture of preserves requires anything between 10–50 m^3 of water per tonne of primary materials consumed and the brewing industry takes 4–15 m^3 of water per tonne of finished beer produced (European Union 2001b). A significant proportion of the water is used for washing purposes and thus the industry as a whole produces relatively large volumes of effluent, which though not generally dangerous to human health or the environment, is heavily loaded with organic matter.

The alternative options to land spreading involve either dedicated on-site treatment or export to an existing local sewage treatment works for coprocessing with domestic wastewater. The choice between them is, of course, largely dictated by commercial concerns though the decision to install an on-site facility, tanker away to another plant or land spread, is often not solely based on economic factors. Regional agricultural practice also plays an important part, in terms of fertiliser and irrigation requirements as well as with respect to environmental and hydrological considerations. It is, of course, a fundamental necessity that the approach selected can adequately cope with both the physical volume of the maximum effluent output on a daily or weekly basis, and the 'strongest' wastewater quality, since each is likely to vary over the year.

Although it is convenient to consider the food and beverage industry as a single group, the effluent produced is extremely variable in composition, depending on the specific nature of the business and the time of the year. However, there are some consistent factors in these effluents, one of which being their typically heavy potassium load. Much of their nutrient component is relatively readily available both for microbial metabolism and plant uptake, which obviously lends itself to rapid utilisation and in addition, the majority of effluents from this sector are comparatively low in heavy metals. Inevitably, these effluents typically contain high levels of organic matter and nitrogen and, consequently, a low C/N ratio, which ensures that they are broken down very rapidly by soil bacteria under even moderately optimised conditions. However, though this is an obvious advantage in terms of their treatability, the concomitant effect of this additional loading on the local microbiota has already been mentioned. In addition, these effluents may frequently contain heavy sodium and chloride loadings originating from the types of cleaning agents commonly used.

The land application of such liquors requires care since too heavy a dose may lead to damage to the soil structure and an alteration of the osmotic balance. Long-term accumulation of these salts within the soil produces a gradual reduction of fertility and ultimately may prove toxic to plants, if left to proceed unchecked. Moreover, the characteristically high levels of unstabilised organic material present and the resultant low carbon to nitrogen ratio tends to make these effluents extremely malodorous, which may present its own constraints on available options for its treatment. It is inevitable that issues of social acceptability make land spread impossible in some areas and, accordingly, a number of food and drink manufacturers have opted for anaerobic digestion as an on-site treatment for their process liquors. This biotechnology, which is described in greater detail in Chapter 8, is extremely effective at transforming the organic matter into a methane-rich biogas, with a high calorific value which can be of direct benefit to the operation to offset the heating and electrical energy costs. Under this method, the organic content of the effluent is rapidly and significantly reduced, and a minimum of sludge produced for subsequent disposal.

Nitrogenous Wastes

For those effluents, however, which are consigned to land treatment regimes, the fate of nitrogen is of considerable importance. In aerobic conditions, the biological nitrification processes within the soil produce nitrate from ammonia and organic nitrogen, principally by the chemotrophic bacteria, *Nitrosomonas* and *Nitrobacter*, which respectively derive first nitrites and then finally nitrates. The oxidation of ammonia (NH_3) can be represented as:

$$2NH_3 + 3O_2 \longrightarrow 2NO_2^- + 2H^+ + 2H_2O$$

This reaction releases energy which is subsequently used by *Nitrosomonas* to reduce carbon dioxide. A secondary oxidation of the nitrate produced by *Nitrobacter* forms nitrate ions, with energy again being released for use by this bacterium.

$$2NO_2^- + O_2 \longrightarrow 2NO_3^-$$

However, in anoxic conditions nitrate compounds can be reduced to nitrogen gas as a result of the activities of various species of facultative and anaerobic soil bacteria, in which the nitrate ion acts as an alternative electron acceptor to oxygen in respiration, as mentioned in Chapter 2. As a result, it becomes possible to view the interlinked processes of nitrogen losses via volatisation, denitrification and plant uptake as control mechanisms for the nitrogenous component in wastewaters in land applications. Approximately 20–30% of the applied nitrogen is lost in this way, a figure which may rise to as much as 50% under some circumstances,

as factors such as high organic content, fine soil particles and water-logging all provide favourable conditions for denitrification within a soil.

Though amelioration processes involving land spreading or injection clearly have beneficial uses for some kinds of wastewaters, in general effluents, particularly those of industrial origin, require more intensive and engineered solutions. In this respect, whether the liquors are treated on-site by the producers themselves, or are tankered to external works is of little significance, since the techniques involved will be much the same irrespective of where they are applied. The contribution of environmental biotechnologies to the safe management of effluents principally centres on microbial action, either in anaerobic digestion where the carbon element is fully reduced, or in aerobic processes which lead to its oxidation. As has been mentioned earlier, the former is covered elsewhere in this book; the rest of this chapter will largely address the latter.

Aeration

Introducing air into liquid wastes is a well-established technique to reduce pollutant potential and is often employed as an on-site method to achieve discharge consent levels, or reduce treatment costs, in a variety of industrial settings. It works by stimulating resident biomass with an adequate supply of oxygen, while keeping suspended solids in suspension and helping to mix the effluent to optimise treatment conditions, which also assists in removing the carbon dioxide produced by microbial activity. In addition, aeration can have a flocculant effect, the extent of which depends on the nature of the effluent. The systems used fall into one of two broad categories, on the basis of their operating criteria:

- Diffused air systems.
- Mechanical aeration.

This classification is a useful way to consider the methods in common use, though it takes account of neither the rate of oxygen transfer, nor the total dissolved oxygen content, which is occasionally used as an alternative way to define aeration approaches.

Diffused air systems

The liquid is contained within a vessel of suitable volume, with air being introduced at the bottom, oxygen diffusing out from the bubbles as they rise, thus aerating the effluent.

These systems can be categorised on the basis of their bubble size, with the crudest being coarse open-ended pipes and the most sophisticated being specialised fine diffusers. Ultra-fine bubble (UFB) systems maximise the oxygen transfer effect, producing a dense curtain of very small bubbles, which consequently have a large surface area to volume ratio to maximise the diffusion.

Table 6.3 *Horticultural waste process liquor analysis before and after 85-day aeration treatment and the associated percentage reductions achieved*

Determinant	Baseline	Post-treatment	% reduction
pH	5.8	8.8	–
Conductivity @20 °C	6 950	6 320	9.1
BOD total + ATU	15 800	198	98.7
COD	27 200	1 990	92.7
Solids particulate 105 °C	6 200	28	99.5
Total dissolved solids	13 700	293	97.9
Ammoniacal nitrogen	515	316	38.6
Total oxidised nitrogen	1.7	0.3	82.4
Kjeldahl nitrogen	926	435	53.0
Nitrite	0.79	0.04	94.9
Nitrate	0.9	0.3	66.7
Sulphate	194	63.4	67.3

All in mg/l except pH (in pH units) and conductivity (in mS/cm). Results courtesy of Rob Heap, unpublished project report.

The UFB system is the most expensive, both to install in the first place and subsequently to run, as it requires comparatively high maintenance and needs a filtered air supply to avoid air-borne particulates blocking the narrow diffuser pores. Illustrative UFB aeration results, based on operational data, obtained from the amelioration of post-anaerobic digestion liquor from a horticultural waste processing plant, are shown in Table 6.3.

Though the comparatively simple approaches which produce large to medium sized bubbles are the least efficient, they are commonly encountered in use since they offer a relatively inexpensive solution.

Mechanical aeration systems

In this method, a partly submerged mechanically driven paddle mounted on floats or attached to a gantry vigorously agitates the liquid, drawing air in from the surface and the effluent is aerated as the bubbles swirl in the vortex created.

Other variants on this theme are brush aerators, which are commonly used to provide both aeration and mixing in the sewage industry and submerged turbine spargers, which introduce air beneath an impeller, which again mixes as it aerates. This latter approach, shown in Figure 6.2, can be considered as a hybrid between mechanical and diffused systems and though, obviously, represents a higher capital cost, it provides great operational efficiency. A major factor in this is that the impeller establishes internal currents within the tank. As a result the bubbles injected at the bottom, instead of travelling straight up, follow a typically spiral path, which increases their mean transit time through the body of the liquid and hence, since their residence period is lengthened, the overall efficacy of oxygen diffusion increases.

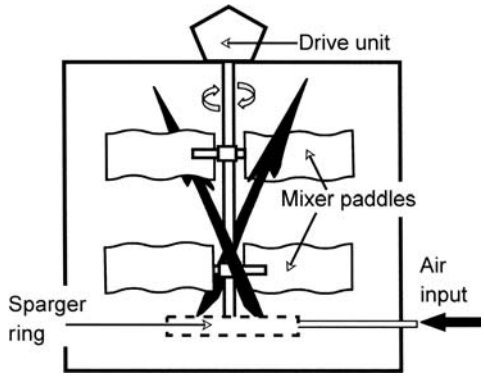

Figure 6.2 *Turbine sparger aeration system*

Table 6.4 *Illustrative oxygen transfer rates for aeration systems at 20°C*

System	Transfer rate ($kg\,O_2/kWh$)
Diffused air	
Coarse bubble	0.6–1.2
Medium bubble	1.0–1.6
Fine bubble	1.2–2.0
Brush aerator	1.2–2.4
Turbine sparger Aerator	1.2–2.4

The design of the system and the processing vessel is crucial to avoid problems of oxygen transfer, liquid stratification and foaming, all of which can be major problems in operation. The time taken to effect treatment depends on the regime used and the nature of the effluent. In this context, Table 6.4 shows typical oxygen transfer rates for aeration systems at 20 °C.

The value of aeration in the treatment process is not restricted to promoting the biological degradation of organic matter, since the addition of oxygen also plays an important role in removing a number of substances by promoting direct chemical oxidation. This latter route can often help eliminate organic compounds which are resistant to straightforward biological treatments.

Trickling Filters

The trickling or biological filter system involves a bed, which is formed by a layer of filter medium held within a containing tank or vessel, often cast from concrete, and equipped with a rotating dosing device, as shown in a stylised form in Figure 6.3.

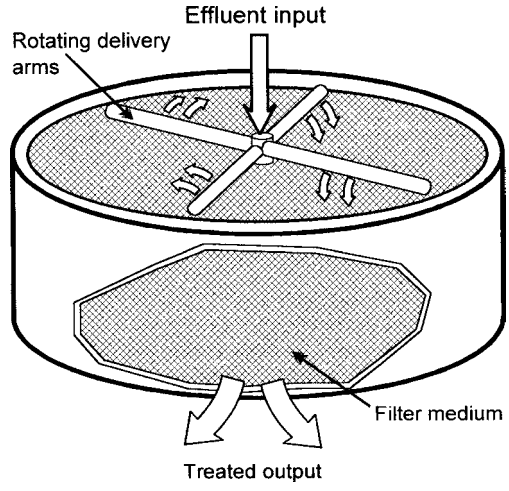

Figure 6.3 *Trickling filter*

The filter is designed to permit good drainage and ventilation and in addition sedimentation and settling tanks are generally associated with the system. Effluent, which has been mechanically cleaned to remove the large particles which might otherwise clog the interparticulate spaces in the filter bed, flows, or is pumped, into the rotating spreader, from which it is uniformly distributed across the filter bed. This dosing process can take place either continuously or intermittently, depending on the operational requirements of the treatment works. The wastewater percolates down through the filter, picking up oxygen as it travels over the surface of the filter medium. The aeration can take place naturally by diffusion, or may sometimes be enhanced by the use of active ventilation fans.

The combination of the available nutrients in the effluent and its enhanced oxygenation stimulates microbial growth, and a gelatinous biofilm of microorganisms forms on the filter medium. This biological mass feeds on the organic material in the wastewater converting it to carbon dioxide, water and microbial biomass. Though the resident organisms are in a state of constant growth, ageing and occasional oxygen starvation of those nearest the substrate leads to death of some of the attached growth, which loosens and eventually sloughs, passing out of the filter bed as a biological sludge in the water flow and thence on to the next phase of treatment.

The filter medium itself is of great importance to the success of these systems and in general the requirements of a good material are that it should be durable and long lasting, resistant to compaction or crushing in use and resistant to frost damage. A number of substances have been used for this purpose including clinker, blast-furnace slag, gravel and crushed rock. A wholly artificial plastic lattice material has also been developed which has proved successful in some

applications, but a clinker and slag mix is generally said to give some of the best results. The ideal filter bed must provide adequate depth to guarantee effluent retention time, since this is critical in allowing it to become sufficiently aerated and to ensure adequate contact between the microbes and the wastewater for the desired level of pollutant removal. It should also have a large surface area for biomass attachment, with generous void spaces between the particles to allow the required biomass growth to take place without any risk of this causing clogging. Finally, it should have the type of surface which encourages splashing on dosing, to entrap air and facilitate oxygenation of the bed.

The trickling filters in use at sewage works are squat, typically around 8–10 metres across and between 1–2 metres deep; though these are the most familiar form, other filters of comparatively small footprint but 5 to 20 metres in height are used to treat certain kinds of trade effluents, particularly those of a stronger nature and with a more heavy organic load than domestic wastewater. They are of particular relevance in an industrial setting since they can achieve a very high throughput and residence time, while occupying a relatively small base area of land.

To maximise the treatment efficiency, it is clearly essential that the trickling filter is properly sized and matched to the required processing demands. The most important factors in arriving at this are the quality of the effluent itself, its input temperature, the composition of the filter medium, detail of the surface-dosing arrangements and the aeration. The wastewater quality has an obvious significance in this respect, since it is this, combined with the eventual clean-up level required, which effectively defines the performance parameters of the system. Although in an ideal world, the filter would be designed around input character, in cases where industrial effluents are co-treated with domestic wastewater in sewage works, it is the feed rate which is adjusted to provide a dilute liquor of given average strength, since the filters themselves are already in existence. Hence, in practice, the load is often adjusted to the facility, rather than the other way about.

The input temperature has a profound influence on the thermal relations within the filter bed, not least because of the high specific heat capacity of water at 4200 J/kg/°C. This can be of particular relevance in industrial reed bed systems, which are discussed in the following chapter, since a warm liquor can help to overcome the problems of cold weather in temperate climes. By contrast the external air temperature appears to have less importance in this respect. The situation within the reaction space is somewhat complicated by virtue of the nonlinear nature of the effect of temperature on contaminant removal. Although the speed of chemical reactions is well known to double for every 10 °C rise, at 20 °C, in-filter biodegradation only represents an increase of 38% over the rate at 10 °C. Below 10 °C, the risk of clogging rises significantly, since the activity of certain key members of the microbial community becomes increasingly inhibited.

The general properties of the filter media were discussed earlier. In respect of sizing the system, the porosity and intergranular spaces govern the interrelation between relative ease of oxygen ingress, wastewater percolation and nutrient to biofilm contact. Clearly, the rougher, pitted or irregular materials tend to offer the greatest surface area per unit volume for microbial attachment and hence, all other things being equal, it follows that the use of such media allows the overall filter dimensions to be smaller. In practice, however, this is seldom a major deciding factor.

In the main, filter systems use rotational dosing systems to ensure a uniform dispersal of the effluent, though nozzles, sprays and mechanised carts are not unknown. The feed must be matched to the medium if the surface aeration effect is to be optimised, but it must also take account of the fluidity, concentration and quality of the wastewater itself and the character of the resident biofilm.

Since the biological breakdown of effluents within the filter is brought about by aerobic organisms, the effectiveness of aeration is of considerable importance. Often adequate oxygenation is brought about naturally by a combination of the surface effects as the wastewater is delivered to the filter, diffusion from atmosphere through the filter medium and an in-filter photosynthetic contribution from algae. Physical air flow due to natural thermal currents may also enhance the oxygenation as may the use of external fans or pumps which are a feature on some industrial units.

Activated Sludge Systems

This approach was first developed in Manchester, just prior to the outbreak of the First World War, to deal with the stronger effluents which were being produced in increasingly large amount by the newly emerging chemicals industry and were proving too toxic for the currently available methods of biological processing. Treatment is again achieved by the action of aerobic microbes, but in this method, they form a functional community held in suspension within the effluent itself and are provided with an enhanced supply of oxygen by an integral aeration system. This is a highly biomass-intensive approach and consequently requires less space than filter to achieve the same treatment. The main features are shown in Figure 6.4.

The activated sludge process has a higher efficiency than the previously described filter system and is better able to adapt to deal with variability in the wastewater input, both in terms of quantity and concentration. However, very great changes in effluent character will challenge it, since the resident microbial community is generally less heterogeneous than commonly found in filters. Additionally, as a more complex system, initial installation costs are higher and it requires greater maintenance and more energy than a trickling filter of comparable throughput.

In use, the sludge tanks form the central part of a three-part system, comprising a settlement tank, the actively aerated sludge vessels themselves and a final

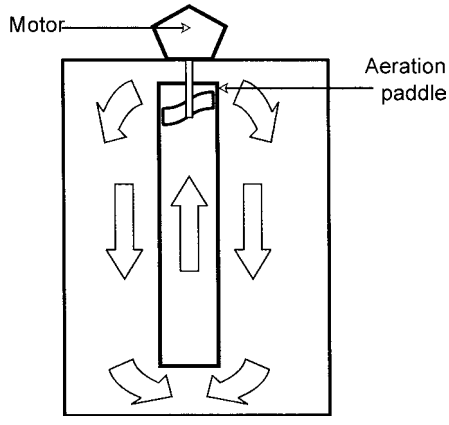

Figure 6.4 *Schematic activated sludge system*

clarifier for secondary sedimentation. The first element of the set-up allows heavy particles to settle at the bottom for removal, while internal baffles or a specifically designed dip pipe off-take excludes floating materials, oil, grease and surfactants.

After this physical pretreatment phase, the wastewater flows into, and then slowly through, the activated sludge tanks, where air is introduced, providing the enhanced dissolved oxygen levels necessary to support the elevated microbial biomass present. These micro-organisms represent a complex and integrated community, with bacteria feeding on the organic content in the effluent, which are themselves consumed by various forms of attached, crawling and free-swimming protozoa, with rotifers also aiding proper floc formation by removing dispersed biomass and the smaller particles which form. The action of aeration also creates a circulation current within the liquid which helps to mix the contents of the tank and homogenise the effluent while also keeping the whole sludge in active suspension. Sludge tanks are often arranged in batteries, so that the part-treated effluent travels though a number of aeration zones, becoming progressively cleaned as it goes.

At the end of the central activated phase, the wastewater, which contains a sizeable sludge component by this stage, leaves these tanks and enters the clarifiers. These are often designed so that the effluent enters at their centre and flows out over a series of weirs along the edge of the clarifier. As the wastewater travels outward, the heavier biological mass sinks to the bottom of the clarifier. Typically, collector arms rotate around the bottom of the tank to collect and remove the settled biomass solids which, since they contain growing bacteria that have developed in the aeration tanks, represent a potentially valuable reservoir of process-acclimatised organisms.

Accordingly, some of this collected biomass, termed the return activated sludge (RAS), is returned to the beginning of the aeration phase to inoculate the new

input effluent. This brings significant benefits to the speed of processing achieved since otherwise, the wastewater would require a longer residence time in which to develop the necessary bacteria and other microbes. It also helps to maintain the high active biomass density which is a fundamental characteristic of this system. The remaining excess sludge is removed for disposal and the clean water flows over another final weir system for discharge, or for tertiary treatment if required.

A similar treatment method sometimes encountered is called aerobic digestion which uses identical vessels to the aeration tanks described, the difference being operational. This involves a batch process approach with a retention period of 30 days or more and since they are not continuously fed, there is no flow-through of liquor within or between digesters. Under these conditions, the bacteria grow rapidly to maturity, but having exhausted the available nutrients, then die off leaving a residue of dead microbial biomass, rather than an activated sludge as before. At the end of the cycle, the contents of the aerobic digesters are transferred to gravity thickeners, which function in much the same way as the secondary clarifiers previously described. The settled solids are returned to the aerobic digester not as an inoculant but as a food source for the next generation, while the clear liquid travels over a separating weir and is returned to the general treatment process.

In effect, then, the 'activated sludge' is a mixture of various micro-organisms, including bacteria, protozoa, rotifers, and higher invertebrate forms, and it is by the combined actions of these organisms that the biodegradable material in the incoming effluent is treated. Thus, it should be obvious that to achieve process control, it is important to control the growth of these microbes, which therefore makes some understanding of the microbiology of activated sludge essential. Bacteria account for around 95% of the microbial mass in activated sludge and most of the dispersed growth suspended in the effluent is bacterial, though ideally there should not be much of this present in a properly operating activated sludge process. Generally speaking this tends only to feature in young sludges, typically less than 3 or 4 days old, and only before proper flocculation has begun. Ciliates are responsible for much of the removal of dispersed growth and adsorption onto the surface of the floc particles themselves also plays a part in its reduction. Significant amounts of dispersed growth characterises the start-up phase, when high nutrient levels are present and the bacterial population is actively growing. However, the presence of excessive dispersed growth in an older sludge can often indicate that the process of proper floc formation has been interrupted in some way. When floc particles first develop they tend to be small and spherical, largely since young sludges do not contain significant numbers of filamentous organisms and those which are present are not sufficiently elongated to aid in the formation process. Thus, the floc-forming bacteria can only flocculate with each other in order to withstand shearing action, hence the typical globular shape. As the sludge ages, the filamentous microbes begin to elongate, their numbers rise and bacterial flocculation occurs along their length, providing greater resistance

to shearing, which in turn favours the floc-forming bacteria. As these thrive and produce quantities of sticky, extracellular slime, larger floc particles are formed, the increasingly irregular shape of which is very apparent on microscopic examination of the activated sludge. Mucus secretions from rotifers, which become more numerous as the sludge ages, also contribute to this overall process. Interruption of this formative succession may occur as a result of high toxicity within the input effluent, the lack of adequate ciliated protozoan activity, excessive inter-tank shearing forces or the presence of significant amounts of surfactant.

Process disruption

Toxicity is a particular worry in the operational plant and can often be assessed by microbiological examination of the sludge. A number of key indicators may be observed which would indicate the presence of toxic components within the system, though inevitably this can often only become apparent after the event. Typically, flagellates will increase in a characteristic 'bloom' while higher life forms, particularly ciliates and the rotifers, die off. The particular sensitivity of these microbe species to toxic inputs has been suggested as a potential method of biomonitoring for toxic stress, but the principle has not yet been developed to a point of practical usefulness.

The floc itself begins to break up as dispersed bacterial growth, characteristic of an immature sludge, returns, often accompanied by foaming within the bioreactor, the progressively reducing growth of microbial biomass leading to a lowered oxygen usage and hence to poor BOD removal. If the toxic event is not so severe as to poison the entire system, as new effluent input washes through the tanks, increasingly diluting the concentration of the contaminating substances and the process recovers, excessive filament formation may occur leading to a condition known as 'filamentous bulking'. As a result, it is sometimes said that toxic inputs favour filamentous bacteria but, with the exception of hydrogen sulphide contamination, this is not strictly true. It is, however, fair to say that the disruption caused by a toxic influx permits their burgeoning growth, particularly since they are generally the fastest group to recover.

By contrast, 'slime bulking' can often occur in industrial activated sludge settings, where the effluent may commonly be deficient in a particular nutrient, most typically either nitrogen or phosphorus. This results in altered floc formation, reduced settling properties and, in some cases, the production of the slimy, greyish foam at the surface of the aeration vessel, which gives this event its name. This greasy, extracellular polymer interferes with the normal settling processes, altering the sludge buoyancy by entrapping air and encouraging foaming. The situation can generally be managed simply by adding appropriate quantities of the missing nutrient, though where relatively easily biodegradable soluble BOD is readily available, it may be necessary to deliberately create higher levels of nitrogen and phosphorus within the system than a straightforward analysis might otherwise indicate.

Foaming can be a significant and unsightly nuisance in operational facilities and, as has been discussed, may occur as a result of either nutrient deficiency or the growth of specific foam-generating filamentous organisms. Microscopic examination of the fresh foam is often the best way to determine which, and thus what remedial action is necessary.

Typical protozoans present in the sludge include amoebae, ciliates and flagellates and, together with rotifers, they play secondary roles in the activated sludge treatment of wastewaters. The presence or absence of particular types can be used as valuable biological indicators of effluent quality or plant performance. In this way, the incidence of large numbers of amoeba often suggests that a shock loading has taken place, making large quantities of food available within the system, or that the dissolved oxygen levels in the tanks have fallen, since they are better able to tolerate conditions of low aeration. A large flagellate population, particularly in mature sludges, suggests the persistence of appreciable quantities of available organic nutrients, since their numbers are usually limited by competition with bacteria for the same dissolved foodstuff. Since ciliates, like rotifers, feed on bacteria, their presence indicates a healthy sludge, as they typically blossom after the floc has been formed and when most of the effluent's soluble nutrients have been removed. As protozoa are more sensitive to pH than floc-forming bacteria, with a typical optimum range of 7.0–7.4 and tolerating 6.0–8.0, they can also provide a broad measure of this parameter in the system.

The population of rotifers seldom approaches large numbers in activated sludge processes, though they nevertheless perform an important function. Their principal role is the removal of dispersed bacteria, thus contributing to both the proper development of floc and the reduction of wastewater turbidity. Taking the longest time of all members of the microbial community to become established in the sludge, their presence indicates increasing stabilisation of the organic components of the effluent.

Organic loadings

Calculating the organic loadings for a given activated sludge system is an important aspect of process control. Measuring the BOD of the incoming wastewater gives a value for the amount of biodegradable matter available for microbial use, which can be used together with an estimate of the resident biomass to derive a relationship termed the food to micro-organism (F/M) ratio. This, which is also sometimes known as the organic loading rate, is given as follows:

$$\text{F/M} = \frac{\text{mass of BOD applied to the biological phase each day}}{\text{total microbial biomass in the biological phase}}$$

The F/M ratio is a useful indication of anticipated micro-organism growth and condition, a high F/M value yielding rapid biomass increase, while a low one suggests little available nutrients and consequently slow growth results. Clearly,

the total active biomass content in an activated sludge system, which is termed the mixed liquor suspended solids (MLSS), is an important factor in process efficacy. Accordingly, it is routinely measured at sewage works being important in the calculation of the F/M ratio, which can be more properly defined as:

$$\text{F/M} = \frac{\text{flow rate}(\text{m}^3/\text{d}) \times \text{BOD}(\text{kg}/\text{m}^3)}{\text{volume of sludge tank}(\text{m}^3) \times \text{MLSS}(\text{kg}/\text{m}^3)}$$

Although the preceding systems are the most common forms likely to be seen in use, a number of other systems exist which may sometimes be encountered, some of which will be briefly outlined for the sake of completeness.

Deep Shaft Process

In many respects this is an activated sludge derivative, which was borne out of ICI's work on the production of proteins from methanol in the 1970s. Figure 6.5 shows the main features of the system, which is based around a shaft 50–100 metres deep.

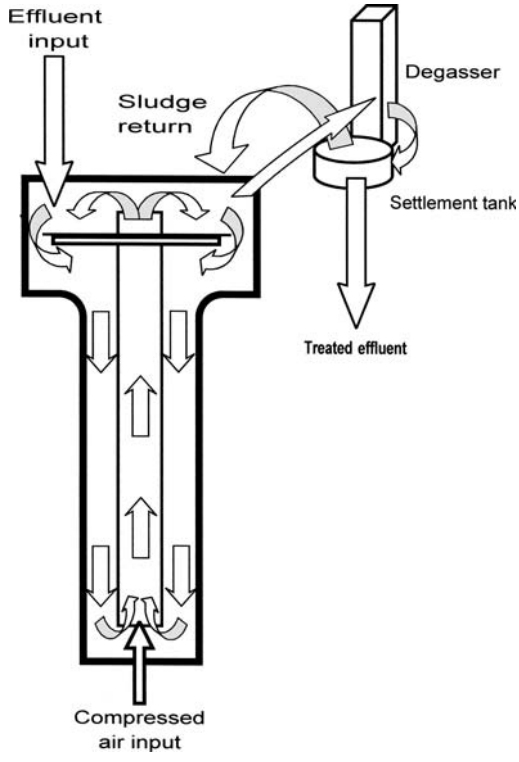

Figure 6.5 *ICI deep shaft process*

The shaft contains the wastewater to be treated, compressed air being blown in at the base, which travels up the central section, setting up an opposing counter flow in the outer part of the shaft. Screened secondary effluent is allowed to settle and a portion of the sludge produced is returned to the input zone, just as in a traditional activated sludge tank, though degassing is required to remove nitrogen and carbon dioxide bubbles from the floc to allow for proper sedimentation.

The high pressures at the base force far more oxygen into solution than normal, which aids aeration enormously and allows the process to achieve an oxygen utilisation of around 90%, which is some 4.5 times better than conventional activated sludge systems. The bubble contact time produced, averaging 90 seconds or more, is over 6 times longer than in standard diffused air systems. It has a low footprint, making it ideal for use in restricted areas.

Pure Oxygen Systems

With process efficacy so closely dependent on aeration and the ability to support a high microbial biomass, the use of pure oxygen to enhance the effective levels of the gas dissolved in the effluent has an obvious appeal. The UNOX™ process, which was developed by the Union Carbide Corporation is probably amongst the best known of the pure oxygen activated sludge systems and Figure 6.6 shows the general layout of the bioreactor vessels.

Pure oxygen obviously gives a better oxygen transfer rate per unit volume of the bioreactor than can be achieved using conventional aeration methods. In turn, this allows a heavier organic loading per unit volume to be treated compared with ordinary air-fed systems, which enables this system to be used to deal with stronger effluents and permits a high throughput where space is restricted. Typically these systems are fed using liquid oxygen tanks.

Despite their clear advantages, pure oxygen systems suffer with some major drawbacks. For one thing, the capital costs involved in installing them in the first place are considerable, as are their running costs and maintenance requirement. The pure oxygen itself represents an explosion risk, thus necessitating intrinsically

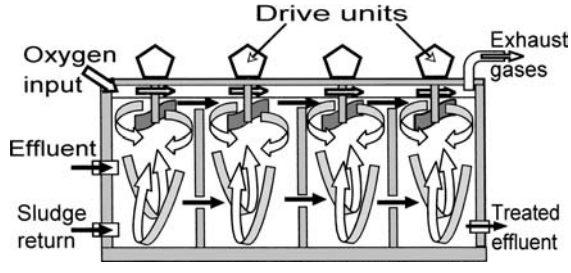

Figure 6.6 *The UNOX™ pure oxygen system*

safe operational procedures and, in addition, leads to accelerated corrosion of the equipment used. However, for some applications and for certain kinds of effluents, they can prove particularly appropriate.

The Oxidation Ditch

This is a sometimes used for sewage treatment and is characterised by a constructed ellipsoidal ditch, in which the effluent is forced to circulate around the channel by brush aerators. The ditch itself is trapezoidal in cross-section to maintain uniform effluent velocity throughout the channel. Effluent is fed into the system without any prior primary sedimentation and typically gives rise to only 50% of the surplus sludge produced by a typical activated sludge process.

The Rotating Biological Contactor

This system, shown in Figure 6.7, is a derivative of the biological filter. It effectively combines the advantages of this previously described approach, like the absence of a complicated settlement system for sludge return and a low maintenance requirement with the smaller footprint and long microbial exposure characteristic of the active sludge process.

They have submerged internal disc baffles which act as sites for the attached growth of biomass, which are slowly turned by electric motor causing the microbes to be alternately aerated and immersed in the effluent. Rotating biological contactors are typically used for small installations, and are particularly useful for applications with high seasonal variations like caravan site sewage systems.

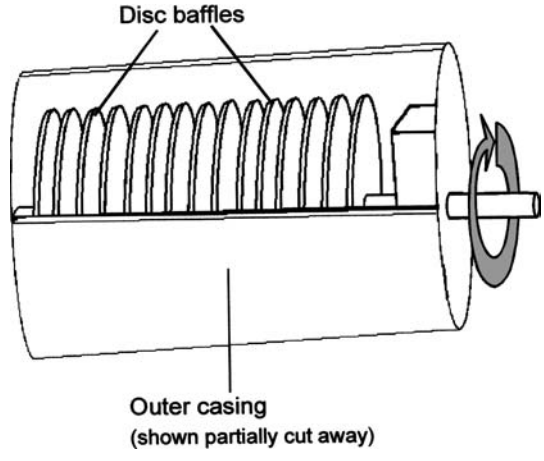

Figure 6.7 *Rotating biological contactor*

Membrane Bioreactors

This system, instead of utilising conventional methods of gravity settlement, achieves the desired biomass retention by means of a cross-flow filtration process, as shown in Figure 6.8.

The development of effective methods of micro- and ultra-filtration has opened up the potential for using membrane bioreactor technology on various forms of domestic and industrial effluents. There are three general types of reactor systems which have been developed, namely solid/liquid separation, gas permeable and extractive systems. The membrane element allows the passage of small molecules, but retains the total resident microbial population. As a result, the cumulative overall bioactivity and the resultant speed of remediation is boosted, since not only are micro-organisms no longer lost with wash-out flow, but also, conditions for even the slowest-growing member species of the microbial community are able to be adequately enhanced. This is of particular relevance to xenobiotics and the more recalcitrant components of wastewaters, as their biological break-down is often brought about by bacteria which themselves have a relatively long establishment period within the population. The high biomass levels within the bioreactor itself obviously necessitate abundant readily available oxygen, though the high organic loading and efficient intrasystem microbial conservation combine to make the hydraulic retention time entirely independent of the solids

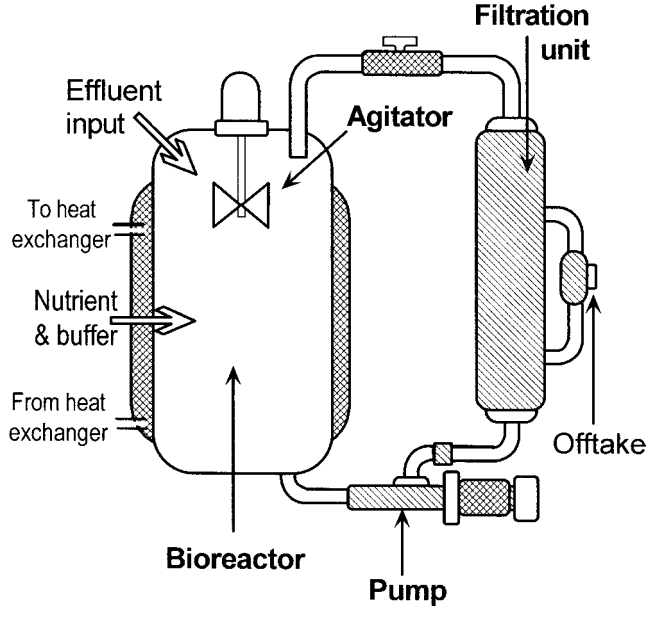

Figure 6.8 *Schematic membrane bioreactor*

themselves. Since the membrane allows gaseous transport while constraining the biological phase, there is provision within the reactor for bubbleless aeration and oxygenation consequently can take place over a relatively large surface area, thereby improving the efficiency of this process. In addition, the membrane itself may become an attachment zone for biofilm formation.

Thus, the membrane bioreactor can offer a greater degradation capacity for persistent chemicals, making possible the biological removal of benzene, nitrobenzene, dichloroaniline and polyaromatic hydrocarbons (PAHs), for example, which represent a significant risk, both to the environment and human health, due to their high toxicity. Removal efficiency for these substances can approach 99%.

The membrane bioreactor has proved its suitability as an efficient system for degradation of recalcitrant compounds and significantly higher biomass concentrations and utilisation rates are routinely achieved than in corresponding alternative treatment systems. In common with most operational, rather than experimental, biological detoxification processes, not all of the contaminants present in the effluent are typically completely converted into carbon dioxide and water, a certain percentage being turned into metabolic byproducts instead, though this can amount to less than 5% in a well-managed bioreactor system. Part of this involves the gradual and controlled introduction of novel wastewater elements, to ensure that acclimatisation is maximised and any potential tendency for 'shock loadings' avoided. This is a clear example of the value of permitting optimised microbial adaptation to the individual application.

These systems are, of course, more expensive than the conventional activated sludge or trickling filters, but produce a much smaller quantity of excess sludge for subsequent disposal of treatment. In addition, they produce an elevated COD removal and would seem particularly well suited to use in small-scale plants where the production of high-quality final effluent is a priority.

Cellulose Ion-Exchange Media

For effluents requiring a highly selective removal of high molecular weight proteins, cellulose ion-exchange media provide an example of treatment involving the use of isolated biologically derived materials. The ion exchange medium is replenished with brine as required, and the proteins collected are removed in the resulting saline solution, for subsequent coagulation and drying. This enables a valuable material to be recovered, typically for use as an animal foodstuff, while reducing the wastewater BOD by 90% or more.

Sludge Disposal

Many of the treatment processes described in this chapter give rise to primary or secondary sludges. Typically, these byproducts require disposal and, like many

forms of solid waste, a proportion have been consigned to either landfill or incineration. For some treated sludges, especially those derived from domestic sewage or food residuals, agricultural use has been an option, often requiring additional treatments to ensure its freedom from human pathogens, before land spreading or injection beneath the surface. The effectiveness of microbes in metal sequestration means, inevitably, that most treated sludges have a degree of heavy metal contamination, which itself makes possible the accumulation of these contaminants in soils exposed to these products. In addition, there are increasingly stringent controls on the release of nitrogen to the environment, particularly within escalating European Union legislation regarding nitrogen vulnerable zones. It would seem, then, that the future land use of 'spent' sludges is likely to be somewhat more heavily regulated than previously.

Closing Remarks

In many respects, the treatment of effluents by biological means is of particular importance to any consideration of environmental biotechnology, since it represents the central point of the previously mentioned intervention triangle, having simultaneous relevance to manufacturing, waste management and pollution control. The majority of manufacturing companies produce wastewaters that contain organic contaminants of one form or another and the traditional route previously in common use: discharge to sewer, watercourse or the sea, is becoming less attractive due to environmental legislation and rising disposal costs. As a result, for an increasing number of companies there is a growing requirement to treat their own effluents and biotechnological processes can often prove the most cost-effective means to achieve this goal.

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Case Study 6.1 Mollusc Biofilters (Rhode Island, USA)

Maintaining or improving the quality of coastal water, particularly around areas of high tourist or amenity value is an increasing concern in many parts of the world. While restricting polluting discharges at their source remains the ideal solution to the problem, various biological interventions have been suggested to deal with the problem directly and the potential of some of these may be developed in future years.

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One particularly interesting example, put forward by the University of Rhode Island, involves utilising the intrinsic abilities of bivalve molluscs such as clams, mussels and oysters, to act as natural biofilters of estuarine waters. These species derive most of their nutritional requirements by sifting the water, actively discriminating between particles, ingesting food while depositing rejects onto the substrate. There is also evidence to suggest that microbial and chemical processes in the sediment convert a number of substances present in this matter to less biologically active forms.

Increasing the local population of suitable shellfish, possibly in artificial aquaculture units, could contribute to a significant reduction in turbidity and improved removal of nitrogen and other nutrients. One of the main advantages of this approach would be that the waters are continually cleaned and at little or no cost. In addition, the system might also be harnessed to offer a boost to the local economy, providing a crop to be harvested or potentially supporting larger commercial ventures.

Even for areas where it would be inappropriate to establish intensive shell fisheries of this kind, initiatives to restore and conserve indigenous mollusc species could prove a cost-effective complement to conservation projects or coastal management strategies, especially those designed to mitigate the impacts of eutrophication.

Case Study 6.2 Flexible Sewage Treatment (Takoradi, Ghana)

Often one of the key features of biological effluent treatment is its simplicity. As the Takoradi raw sewage treatment plants in Ghana show, for many parts of the world, when resources or skilled labour are in short supply, this can be a major advantage.

The work, which involved dealing with the effluent from military barracks, was carried out by Naston of Weybridge, England. Both of the systems supplied were designed on the basis of a population equivalent of 5000, with a daily throughput of up to 1 million litres. Each site was equipped with twin aeration tanks 19 metres in diameter, containing integral settlement chambers, aeration and excess sludge removal systems, together with associated 6 metre diameter sludge-holding tanks. The use of two treatment cells, rather than a single, large one, enables the plant to be operated for maximum efficiency, allowing one of them to be shut down if the population falls below half its design capacity for extended periods.

Since both facilities are in remote areas and were built under supervision by unskilled labour, the design deliberately minimised the civil engineering requirement. Other aspects of the plants were, likewise, kept simple to ensure that the system is easy to operate, involving no daily maintenance and little more than a routine periodic servicing to the pumps and aerators.

Thus the wastewater collects at the pumping facilities and is transferred directly to the aeration tanks, where it is processed by the rich complement of microorganisms growing in the well-oxygenated environment. After the aeration phase, the mixed liquor of treated sewage and active microbes is transferred at a constant rate to the clarifiers, which helps to achieve a good separation and the resulting

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offtake is typically of high quality. The excess biomass arising is dewatered and tankered off site, while the supernatant liquor is returned as an inoculant to the aeration tank.

It is a particularly simple system in principle and clearly illustrates that practical 'biotech' solutions do not have to be technologically complex or highly engineered. However, operational success is only possible given a thorough appreciation of the biological processes involved and the constraints imposed by particular local circumstances.

7

Phytotechnology and Photosynthesis

From a practical standpoint, phytotechnology is the use of plants in environmental biotechnology applications, and draws on many of the characteristics which have already been described. In this respect, it does not represent a single unified technology, or even application, but rather is a wider topic, defined solely by the effector organisms used. Thus the fundamental scope of this chapter is broader and the uses and mechanisms described somewhat more varied than for many of the preceding biotechnologies discussed.

Plants of one kind or another can be instrumental in the biological treatment of a large number of substances which present many different types of environmental challenges. Accordingly, they may be used to remediate industrial pollution, treat effluents and wastewaters or solve problems of poor drainage or noise nuisance. The processes of bioaccumulation, phytoextraction, phytostabilisation and rhizofiltration are collectively often referred to as phytoremediation. Although it is sometimes useful to consider them separately, in most functional respects, they are all aspects of the same fundamental plant processes and hence there is much merit in viewing them as parts of a cohesive whole, rather than as distinctly different technologies. It is important to be aware of this, particularly when reading a variety of other published accounts, as the inevitable similarities between descriptions can sometimes lead to confusion. Moreover, the role of phyto*technology* is not limited solely to phyto*remediation* and this discussion, as explained above, is more deliberately inclusive of wider plant-based activities and uses.

Despite the broad spectrum of potential action exhibited by plants in this respect, there are really only three basic mechanisms by which they achieve the purpose desired. In essence, all phytotechnology centres on the removal and accumulation of unwanted substances within the plant tissues themselves, their removal and subsequent volatisation to atmosphere or the facilitation of in-soil treatment. Plant-based treatments make use of natural cycles within the plant and its environment and, clearly, to be effective, the right plant must be chosen. Inevitably, the species selected must be appropriate for the climate, and it must, obviously, be able to survive in contact with the contamination to be able to accomplish its goal. It may also have a need to be able to encourage localised microbial growth.

One of the major advantages of phytotechnological interventions is their almost universal approval from public and customer alike and a big part of the appeal lies in the aesthetics. Healthy plants, often with flowers, makes the site look more attractive, and helps the whole project be much more readily accepted by people who live or work nearby. However, the single biggest factor in its favour is that plant-based processes are frequently considerably cheaper than rival systems, so much so that sometimes they are the only economically possible method. Phytoremediation is a particularly good example of this, especially when substantial areas of land are involved. The costs involved in cleaning up physically large contamination can be enormous and for land on which the pollution is suitable and accessible for phytotreatment, the savings can be very great. Part of the reason for this is that planting, sowing and harvesting the relevant plants requires little more advanced technology or specialised equipment than is readily at the disposal of the average farmer.

The varied nature of phytotechnology, as has already been outlined, makes any attempt at formalisation inherently artificial. However, for the purposes of this discussion, the topic will be considered in two general sections, purely on the basis of whether the applications themselves represent largely aquatic or terrestrial systems. The reader is urged to bear in mind that this is merely a convenience and should be accorded no particular additional importance beyond that.

Terrestrial Phyto-Systems (TPS)

The importance of pollution, contaminated land and the increasing relevance of bioremediation have been discussed in previous chapters. Phytoremediation methods offer significant potential for certain applications and, additionally, permit much larger sites to be restored than would generally be possible using more traditional remediation technologies. The processes of photosynthesis described earlier in this chapter are fundamental in driving what is effectively a solar-energy driven, passive and unengineered system and hence may be said to contribute directly to the low cost of the approach.

A large range of species from different plant groups can be used, ranging from pteridophyte ferns, to angiosperms like sunflowers, and poplar trees, which employ a number of mechanisms to remove pollutants. There are over 400 different species considered suitable for use as phytoremediators. Amongst these, some hyperaccumulate contaminants within the plant biomass itself, which can subsequently be harvested, others act as pumps or siphons, removing contaminants from the soil before venting them into the atmosphere, while others enable the biodegradation of relatively large organic molecules, like hydrocarbons derived from crude oil. However, the technology is relatively new and so still in the development phase. The first steps toward practical bioremediation using various plant-based methods really began with research in the early 1990s and a number of the resulting techniques have been used in the field with reasonable success.

In effect, phytoremediation may be defined as the direct *in situ* use of living green plants for treatment of contaminated soil, sludges or groundwater, by the removal, degradation, or containment of the pollutants present. Such techniques are generally best suited to sites on which low to moderate levels of contamination are present fairly close to the surface and in a relatively shallow band. Within these general constraints, phytoremediation can be used in the remediation of land contaminated with a variety of substances including certain metals, pesticides, solvents and various organic chemicals.

Metal Phytoremediation

The remediation of sites contaminated with metals typically makes use of the natural abilities of certain plant species to remove or stabilise these chemicals by means of bioaccumulation, phytoextraction, rhizofiltration or phytostabilisation.

Phytoextraction

The process of phytoextraction involves the uptake of metal contaminants from within the soil by the roots and their translocation into the above-ground regions of the plants involved. Certain species, termed hyperaccumulators, have an innate ability to absorb exceptionally large amounts of metals compared to most ordinary plants, typically 50–100 times as much (Chaney *et al*. 1997, Brooks *et al*. 1998) and occasionally considerably more. The original wild forms are often found in naturally metal-rich regions of the globe where their unusual ability is an evolutionary selective advantage. Currently, the best candidates for removal by phytoextraction are copper, nickel and zinc, since these are the metals most readily taken up by the majority of the varieties of hyperaccumulator plants. In order to extend the potential applicability of this method of phytoremediation, plants which can absorb unusually high amounts of chromium and lead are also being trialled and there have been some recent early successes in attempts to find suitable phytoextractors for cadmium, nickel and even arsenic. The removal of the latter is a big challenge, since arsenic behaves quite differently from other metal pollutants, typically being found dissolved in the groundwater in the form of arsenite or arsenate, and does not readily precipitate. There have been some advances like the application of bipolar electrolysis to oxidise arsenite into arsenate, which reacts with ferric ions from an introduced iron anode, but generally conventional remediation techniques aim to produce insoluble forms of the metal's salts, which, though still problematic, are easier to remove. Clearly, then, a specific arsenic-tolerant plant selectively pulling the metal from the soil would be a great breakthrough. One attempt to achieve this which has shown some promise involves the Chinese ladder brake fern, *Pteris vittata*, which has been found to accumulate arsenic in concentrations of 5 grams per kilogramme of dry biomass. Growing very rapidly and amassing the metal in its root and stem tissue, it is easy to harvest for contaminant removal.

Hyperaccumulation

Hyperaccumulation itself is a curious phenomenon and raises a number of fundamental questions. While the previously mentioned pteridophyte, *Pteris vittata*, tolerates tissue levels of 0.5% arsenic, certain strains of naturally occurring alpine pennycress (*Thlaspi caerulescens*) can bioaccumulate around 1.5% cadmium, on the same dry weight basis. This is a wholly exceptional concentration. Quite how the uptake and the subsequent accumulation is achieved are interesting enough issues in their own right. However, more intriguing is why so much should be taken up in the first place. The hyperaccumulation of copper or zinc, for which there is an underlying certain metabolic requirement can, to some extent, be viewed as the outcome of an over-efficient natural mechanism. The biological basis of the uptake of a completely nonessential metal, however, particularly in such amounts, remains open to speculation at this point. Nevertheless, with plants like *Thlaspi* showing a zinc removal rate in excess of 40 kg per hectare per year, their enormous potential value in bioremediation is very clear.

In a practical application, appropriate plants are chosen based on the type of contaminant present, the regional climate and other relevant site conditions. This may involve one or a selection of these hyperaccumulator species, dependent on circumstances. After the plants have been permitted to grow for a suitable length of time, they are harvested and the metal accumulated is permanently removed from the original site of contamination. If required, the process may be repeated with new plants until the required level of remediation has been achieved. One of the criticisms commonly levelled at many forms of environmental biotechnology is that all it does is shift a problem from one place to another. The fate of harvested hyperaccumulators serves to illustrate the point, since the biomass thus collected, which has bioaccumulated significant levels of contaminant metals, needs to be treated or disposed of itself, in some environmentally sensible fashion. Typically the options are either composting or incineration. The former must rely on co-composting additional material to dilute the effect of the metal-laden hyperaccumulator biomass if the final compost is to meet permissible levels; the latter requires the ash produced to be disposed of in a hazardous waste landfill. While this course of action may seem a little unenvironmental in its approach, it must be remembered that the void space required by the ash is only around a tenth of that which would have been needed to landfill the untreated soil.

An alternative that has sometimes been suggested is the possibility of recycling metals taken up in this way. There are few reasons, at least in theory, as to why this should not be possible, but much of the practical reality depends on the value of the metal in question. Dried plant biomass could be taken to processing works for recycling and for metals like gold, even a very modest plant content could make this economically viable. By contrast, low value materials, like lead for example, would not be a feasible prospect. At the moment, nickel is probably the best studied and understood in this respect. There has been considerable interest in the potential for biomining the metal out of sites which have

been subject to diffuse contamination, or former mines where further traditional methods are no longer practical. The manner proposed for this is essentially phytoextraction and early research seems to support the economic case for drying the harvested biomass and then recovering the nickel. Even where the actual post-mining residue has little immediate worth, the application of phytotechnological measures can still be of benefit as a straightforward clean-up. In the light of recent advances in Australia, using the ability of eucalyptus trees and certain native grasses to absorb metals from the soil, the approach is to be tested operationally for the decontamination of disused gold mines (Murphy and Butler 2002). These sites also often contain significant levels of arsenic and cyanide compounds. Managing the country's mining waste is a major expense, costing in excess of Aus$30 million per year; success in this trial could prove of great economic advantage to the industry.

The case for metals with intermediate market values is also interesting. Though applying a similar approach to zinc, for instance, might not result in a huge commercial contribution to the smelter, it would be a benefit to the metal production and at the same time, deal rationally with an otherwise unresolved disposal issue. Clearly, the metallurgists would have to be assured that it was a worthwhile exercise. The recycling question is a long way from being a workable solution, but potentially it could offer a highly preferable option to the currently prevalent landfill route.

Rhizofiltration

Rhizofiltration is the absorption into, or the adsorption or precipitation onto, plant roots of contaminants present in the soil water. The principal difference between this and the previous approach is that rhizofiltration is typically used to deal with contamination in the groundwater, rather than within the soil itself, though the distinction is not always an easy one to draw. The plants destined to be used in this way are normally brought on hydroponically and gradually acclimatised to the specific character of the water which requires to be treated. Once this process has been completed, they are planted on the site, where they begin taking up the solution of pollutants. Harvesting takes place once the plants have become saturated with contaminants and, as with the phytoextraction, the collected biomass requires some form of final treatment. The system is less widely appreciated than the previous technology, but it does have some very important potential applications. Sunflowers were reported as being successfully used in a test at Chernobyl in the Ukraine, to remove radioactive uranium contamination from water in the wake of the nuclear power station accident.

Phytostabilisation

In many respects, phytostabilisation has close similarities with both phytoextraction and rhizofiltration in that it too makes use of the uptake and accumulation by,

adsorption onto, or precipitation around, the roots of plants. On first inspection, the difference between these approaches is difficult to see, since in effect, phytostabilisation does employ both extractive and filtrative techniques. However, what distinguishes this particular phytoremediation strategy is that, unlike the preceding regimes, harvesting the grown plants is not a feature of the process. In this sense, it does not remove the pollutants, but immobilises them, deliberately concentrating and containing them within a living system, where they subsequently remain. The idea behind this is to accumulate soil or groundwater contaminants, locking them up within the plant biomass or within the rhizosphere, thus reducing their bio-availability and preventing their migration off site. Metals do not ultimately degrade, so it can be argued that holding them in place in this way is the best practicable environmental option for sites where the contamination is low, or for large areas of pollution, for which large-scale remediation by other means would simply not be possible.

A second benefit of this method is that on sites where elevated concentrations of metals in the soil inhibits natural plant growth, the use of species which have a high tolerance to the contaminants present enables a cover of vegetation to be re-established. This can be of particular importance for exposed sites, minimising the effects of wind erosion, wash off or soil leaching, which otherwise can significantly hasten the spread of pollutants around and beyond the affected land itself.

Organic Phytoremediation

A wide variety of organic chemicals are commonly encountered as environmental pollutants including many types of pesticides, solvents and lubricants. Probably the most ubiquitous of these across the world, for obvious reasons, are petrol and diesel oil. These hydrocarbons are not especially mobile, tend to adhere closely to the soil particles themselves and are generally localised within 2 metres of the surface. Accordingly, since they are effectively in direct contact with the rhizosphere, they are a good example of ideal candidates for phytoremediation. The mechanisms of action in this respect are typically phytodegradation, rhizodegradation, and phytovolatilisation.

Phytodegradation

Phytodegradation, which is sometimes known by the alternative name of phytotransformation, involves the biological breakdown of contaminants, either internally, having first been taken up by the plants, or externally, using enzymes secreted by them. Hence, the complex organic molecules of the pollutants are subject to biodegradation into simpler substances and incorporated into the plant tissues. In addition, the existence of the extracellular enzyme route has allowed this technique to be successfully applied to the remediation of chemicals as varied as chlorinated solvents, explosives and herbicides. Since this process depends

on the direct uptake of contaminants from soil water and the accumulation of resultant metabolites within the plant tissues, in an environmental application, it is clearly important that the metabolites which accumulate are either nontoxic, or at least significantly less toxic than the original pollutant.

Rhizodegradation

Rhizodegradation, which is also variously described as phytostimulation or enhanced rhizospheric biodegradation, refers to the biodegradation of contaminants in the soil by edaphic microbes enhanced by the inherent character of the rhizosphere itself. This region generally supports high microbial biomass and consequently a high level of microbiological activity, which tends to increase the speed and efficiency of the biodegradation of organic substances within the rhizosphere compared with other soil regions and microfloral communities. Part of the reason for this is the tendency for plant roots to increase the soil oxygenation in their vicinity and exude metabolites into the rhizosphere. It has been estimated that the release of sugars, amino acids and other exudates from the plant and the net root oxygen contribution can account for up to 20% of plant photosynthetic activity per year (Foth 1990), of which denitrifying bacteria, *Pseudomonas spp*., and general heterotrophs are the principal beneficiaries. In addition, mycorrhizae fungi associated with the roots also play a part in metabolising organic contaminants. This is an important aspect, since they have unique enzymatic pathways that enable the biodegradation of organic substances that could not be otherwise transformed solely by bacterial action. In principle, rhizodegradation is intrinsic remediation enhanced by entirely natural means, since enzymes which are active within 1 mm of the root itself, transform the organic pollutants, in a way which, clearly, would not occur in the absence of the plant. Nevertheless, this is generally a much slower process than the previously described phytodegradation.

Phytovolatilisation

Phytovolatilisation involves the uptake of the contaminants by plants and their release into the atmosphere, typically in a modified form. This phytoremediation biotechnology generally relies on the transpiration pull of fast-growing trees, which accelerates the uptake of the pollutants in groundwater solution, which are then released through the leaves. Thus the contaminants are removed from the soil, often being transformed within the plant before being voided to the atmosphere. One attempt which has been explored experimentally uses a genetically modified variety of the Yellow Poplar, *Liriodendron tulipifera*, which has been engineered by the introduction of mercuric reductase gene (*mer A*) as discussed in Chapter 9. This confers the ability to tolerate higher mercury concentrations and to convert the metal's ionic form to the elemental and allows the plant to withstand contaminated conditions, remove the pollutant from the soil and

volatilise it. The advantages of this approach are clear, given that the current best available technologies demand extensive dredging or excavation and are heavily disruptive to the site.

The choice of a poplar species for this application is interesting, since they have been found useful in similar roles elsewhere. Trichloroethylene (TCE), an organic compound used in engineering and other industries for degreasing, is a particularly mobile pollutant, typically forming plumes which move beneath the soil's surface. In a number of studies, poplars have been shown to be able to volatilise around 90% of the TCE they take up. In part this relates to their enormous hydraulic pull, a property which will be discussed again later in this chapter. Acting as large, solar-powered pumps, they draw water out of the soil, taking up contaminants with it, which then pass through the plant and out to the air.

The question remains, however, as to whether there is any danger from this kind of pollutant release into the atmosphere and the essential factor in answering that must take into account the element of dilution. If the trees are pumping out mercury, for instance, then the daily output and its dispersion rate must be such that the atmospheric dilution effect makes the prospect of secondary effects, either to the environment or to human health, impossible. Careful investigation and risk analysis is every bit as important for phytoremediation as it is for other forms of bioremediation.

Using tree species to clean up contamination has begun to receive increasing interest. Phytoremediation in general tends to be limited to sites where the pollutants are located fairly close to the surface, often in conjunction with a relatively high water table. Research in Europe and the USA has shown that the deeply penetrating roots of trees allows deeper contamination to be treated. Once again, part of the reason for this is the profound effect these plants can have on the local water relations.

Hydraulic Containment

Large plants can act as living pumps, pulling large amounts of water out of the ground which can be a useful property for some environmental applications, since the drawing of water upwards through the soil into the roots and out through the plant decreases the movement of soluble contaminants downwards, deeper into the site and into the groundwater. Trees are particularly useful in this respect because of their enormous transpiration pull and large root mass. Poplars, for example, once established, have very deep tap roots and they take up large quantities of water, transpiring between 200–1100 litres daily. In situations where grassland would normally support a water table at around 1.5 metres, this action can lead to it being up to 10 times lower. The aim of applying this to a contamination scenario is to create a functional water table depression, to which pollutants will tend to be drawn and from which they may additionally be taken up for treatment. This use of the water uptake characteristics of plants to control the migration of

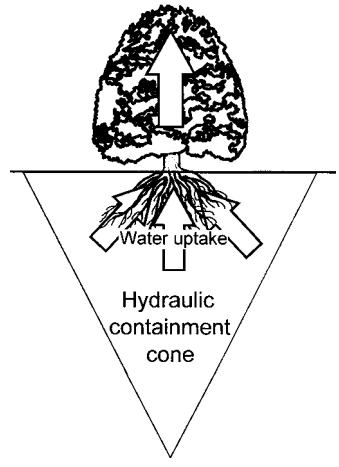

Figure 7.1 *Schematic hydraulic containment*

contaminants in the soil is termed hydraulic containment, shown schematically in Figure 7.1, and a number of particular applications have been developed.

Buffer strips are intended to prevent the entry of contaminants into watercourses and are typically used along the banks of rivers, when they are sometimes called by the alternative name of 'riparian corridors', or around the perimeter of affected sites to contain migrating chemicals. Various poplar and willow varieties, for example, have shown themselves particularly effective in reducing the wash-out of nitrates and phosphates making them useful as pollution control measures to avoid agricultural fertiliser residues contaminating waterways. Part of the potential of this approach is that it also allows for the simultaneous integration of other of the phytoremediating processes described into a natural treatment train, since as previously stated, all plant-based treatments are aspects of the same fundamental processes and thus part of a cohesive whole.

Another approach sometimes encountered is the production of vegetative caps, which has found favour as a means of finishing off some American landfill sites. The principle involves planting to preventing the downward percolation of rainwater into the landfill and thus minimising leachate production while at the same time reducing erosion from the surface. The method seems to be successful as a living alternative to an impermeable clay or geopolymer barrier. The vegetative cap has also been promoted for its abilities to enhance the biological breakdown of the underlying refuse. In this respect, it may be seen as an applied form of rhizodegradation or even, arguably, of phytodegradation. How effective it is likely to be in this role, however, given the great depths involved in most landfills and the functionally anoxic conditions within them, appears uncertain.

To understand the overall phytoremediation effect of hydraulic containment, it is important to realise that contaminating organics are actually taken up by the

plant at lower concentration than they are found *in situ*, in part due to membrane barriers at the root hairs. In order to include this in a predictive mathematical model, the idea of a transpiration stream concentration factor (TSCF) for given contaminants has been developed, defined as $\mathrm{TSCF} = 0.75 \exp\{-[(\log K_{\mathrm{ow}} - 2.50)2/2.4]\}$, (Burken and Schnoor 1998) where K_{ow} is the octanol–water partition coefficients. These latter are a measure of the hydrophobia or hydrophilia of a given organic chemical; a log K_{ow} below 1 characterises the fairly soluble, while above 3.5 indicates highly hydrophobic substances.

Thus the uptake rate (U in mg/day) is given by the following equation:

$$U = (TSCF)TC$$

Where:

$TSCF$ = transpiration stream concentration factor, as defined
T = transpiration rate of vegetation, l/day
C = concentration in site water, mg/l

However, it must also be remembered in this context that, should the pollutants not themselves actually be taken up by the plants, then the effect of establishing a hydraulic containment regime will be to increase their soil concentration due to transpiro-evaporative concentration. Thus, the mass of affected water in the contaminant plume reduces, as does the consequent level of dilution it offers and hence, increased localised concentration can result.

The transpiration pull of plants, and particularly tree species, has also sometimes been harnessed to overcome localised water-logging, particularly on land used for agricultural or amenity purposes. To enhance the effect at the point worst affected, the planting regime may involve the establishment of close groupings, which then function as single elevated withdrawal points. The noted ability of poplars to act as solar-powered hydraulic pumps makes them of great potential benefit to this kind of phytotechnological application. Although other plant-based processes could be taking place at the same time to remediate land alongside this to clean up contaminated soils, this particular technique is not itself a type of phytoremediation. Instead, it is an example of the broader bioengineering possibilities which are offered by the appropriate use of flora species to wider environmental nuisances, which, for some sites, may be the only economic or practicable solution. This may be of particular relevance to heavy soils with poor natural interparticulate spacing, since laying adequate artificial drainage systems can often be expensive to do in the first place and are frequently prone to collapse once installed.

Another similar example of the use of phytotechnology to overcome nuisance is the bio-bund, which consists of densely planted trees, often willows, on an engineered earthwork embankment. This system has been used successfully to reduce noise pollution from roads, railways and noisy industrial sites, the interlocking branches acting as a physical barrier to deaden the sound as well as

having a secondary role in trapping wind-blown particulates. Depending on the individual site, the bio-bund can be constructed in such a way that it can also act as a buffer strip to control migrating chemical pollution, if required.

Plant Selection

It should be obvious that the major criteria for plant selection are the particular requirements for the method to be employed and the nature of the contaminants involved. For example, in the case of organic phytotransformation this means species of vegetation which are hardy and fast growing, easy to maintain, have a high transpiration pull and transform the pollutants present to nontoxic or less toxic products. In addition, for many such applications, deep rooting plants are particularly valuable.

On some sites, the planting of grass varieties in conjunction with trees, often in between rows of trees to stabilise and protect the soil, may be the best route since they generate a tremendous amount of fine roots near to the surface. This particularly suits them to transforming hydrophobic contaminants such as benzene, toluene, ethylbenzene, xylenes (collectively known as BTEX) and polycyclic aromatic hydrocarbons (PAHs). They can also be very helpful in controlling wind-blown dust, wash-off and erosion. The selection of appropriate plant species for bioengineering is not, however, limited solely to their direct ability to treat contaminants, since the enhancement of existing conditions forms as much a part of the potential applications of phytotechnology as bioremediation. For instance, legumes can be of great benefit to naturally nitrogen-deficient soils, since they have the ability, via symbiotic root nodule bacteria, to directly fix nitrogen from the atmosphere. With so much to take into consideration in plant selection, the value of a good botanist or agronomist in any interdisciplinary team is clear.

Applications

Phytotechnology has many potentially beneficial land uses, though for the most part the applications are still in the development stage. Several have been tested for the treatment of contamination, and in some cases successfully tried in the field, but generally they remain in the 'novel and innovative' category, lacking well-documented data on their performance under a variety of typical operating conditions. As a result, some researchers have voiced doubts, suggesting that the beneficial effects of plant utilisation, particularly in respect of phytoremediation, have been overstated. Some have argued that the reality may range from genuine enhancement to no effect, or even to a negative contribution under certain circumstances and that the deciding factors have more to do with the nature of the site than the plants themselves. In addition, some technologies which have been

successfully used on some sites may simply serve to complicate matters on others. One such approach which achieved commercial scale use in the USA, principally for lead remediation, required the addition of chemicals to induce metal take-up. Lead normally binds strongly to the soil particles and so its release was achieved by using chelating agents like ethylene diamine tetra acetic acid (EDTA), which were sprayed onto the ground. With the lead rendered biologically available, it can be taken up by plants and hence removed. However, dependent on the character of the site geology, it has been suggested that this could also allow lead to percolate downwards through the soil, and perhaps ultimately into watercourses. While it may well be possible to overcome this potential problem, using accurate mathematical modelling, followed by the establishment of good hydraulic containment as an adjunct to the process, or by running it in a contained biopile, it does illustrate one of the major practical limitations of plant bioengineering. The potential benefits of phytotechnology for inexpensive, large-scale land management are clear, but the lack of quantitative field data on its efficacy, especially compared with actively managed alternative treatment options, is a serious barrier to its wider adoption. In addition, the roles of enzymes, exudates and metabolites need to be more clearly understood and the selection criteria for plant species and systems for various contamination events requires better codification. Much research is underway in both public and the private sectors which should throw considerable light on these issue. Hopefully it will not be too long in the future before such meaningful comparisons can be drawn.

One area where phytoremediation may have a particular role to play, and one which might be amenable to early acceptance is as a polishing phase in combination with other clean-up technologies. As a finishing process following on from a preceding bioremediation or nonbiological method first used to deal with 'hot-spots', plant-based remediation could well represent an optimal low-cost solution. The tentative beginnings of this have already been tried in small-scale trials and techniques are being suggested to treat deeply located contaminated groundwater by simply pumping to the surface and using it as the irrigant for carefully selected plant species, allowing them to biodegrade the pollutants. The lower levels of site intrusion and engineering required to achieve this would bring clear benefits to both the safety and economic aspects of the remediation operation.

Aquatic Phyto-Systems (APS)

Aquatic phyto-systems are principally used to process effluents of one form or another, though manufactured wetlands have been used successfully to remediate some quite surprising soil contaminants, including TNT residues. Though the latter type of application will be discussed in this section, it is probably best considered as an intergrade between the other APS described hereafter and the TPS of the previous. Many of the aspects of the biotreatment of sewage and

other wastewaters have already been covered in the previous chapter and so will not be restated here. The major difference between conventional approaches to deal with effluents and phytotechnological methods is that the former tend to rely on a faster, more intensively managed and high energy regime, while in general, the stabilisation phase of wastewaters in aquatic systems is relatively slow. The influx and exit of effluent into and out of the created wetland must be controlled to ensure an adequate retention period to permit sufficient residence time for pollutant reduction, which is inevitably characterised by a relatively slow flow rate. However, the efficiency of removal is high, typically producing a final treated off-take of a quality which equals, or often exceeds, that of other systems. Suffice it to say that, as is typical of applications of biological processing in general, there are many common systemic considerations and constraints which will obviously affect phyto-systems, in much the same way as they did for technologies which rely on microbial action for their effect.

Many aquatic plant species have the potential to be used in treatment systems and the biological mechanisms by which they achieve some of the effects will already be largely familiar from the preceding discussion of terrestrial systems. There are a number of ways in which APS can be categorised but perhaps the most useful relates to the natural division between algae and macrophytes, which has been adopted, accordingly, here.

Macrophyte Treatment Systems (MaTS)

The discharge of wastewaters into natural watercourses, ponds and wetlands is an ancient and long-established practice, though rising urbanisation led to the development of more engineered solutions, initially for domestic sewage and then later, industrial effluents, which in turn for a time lessened the importance of the earlier approach. However, there has been a resurgence of interest in simpler, more natural methods for wastewater treatment and MaTS systems, in particular, have received much attention as a result. While there has, undoubtedly, been a strong upsurge in public understanding of the potential for environmentally harmonious water cleaning *per se*, a large part of the driving force behind the newly found interest in these constructed habitats comes from biodiversity concerns. With widespread awareness of the dwindling number of natural wetlands, often a legacy of deliberate land drainage for development and agricultural purposes, the value of such manufactured replacements has become increasingly apparent. In many ways it is fitting that this should be the case, since for the majority of aquatic macrophyte systems, even those expressly intended as 'monocultures' at the gross scale, it is very largely as a result of their biodiversity that they function as they do.

These treatment systems, shown diagrammatically in Figure 7.2, are characterised by the input of effluent into a reservoir of comparatively much larger volume, either in the form of an artificial pond or an expanse of highly saturated soil

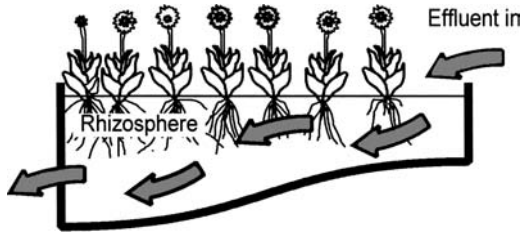

Figure 7.2 *Diagrammatic macrophyte treatment system (MaTS)*

held within a containment layer, within which the macrophytes have been established. Less commonly, pre-existing natural features have been used. Although wetlands have an innate ability to accumulate various unwanted chemicals, the concept of deliberately polluting a habitat by using it as a treatment system is one with which few feel comfortable today. A gentle hydraulic flow is established, which encourages the incoming wastewater to travel slowly through the system. The relatively long retention period that results allows adequate time for processes of settlement, contaminant uptake, biodegradation and phytotransformation to take place.

The mechanisms of pollutant removal are essentially the same, irrespective of whether the particular treatment system is a natural wetland, a constructed monoculture or polyculture and independent of whether the macrophytes in question are submerged, floating or emergent species. Both biotic and abiotic methods are involved. The main biological mechanisms are direct uptake and accumulation, performed in much the same manner as terrestrial plants. The remainder of the effect is brought about by chemical and physical reactions, principally at the interfaces of the water and sediment, the sediment and the root or the plant body and the water. In general, it is possible to characterise the primary processes within the MaTS as the uptake and transformation of contaminants by micro-organisms and plants and their subsequent biodegradation and biotransformation; the absorption, adsorption and ion exchange on the surfaces of plants and the sediment; the filtration and chemical precipitation of pollutants via sediment contact; the settlement of suspended solids; the chemical transformation of contaminants. It has been suggested that although settlement inevitably causes the accrual of metals, in particular, within the sediment, the plants themselves do not tend to accumulate them within their tissues. While this appears to be borne out, particularly by original studies of natural wetlands used for the discharge of mine washings (Hutchinson 1975), this does not form any basis on which to disregard the contribution the plants make to water treatment. For one thing, planting densities in engineered systems are typically high and the species involved tend to be included solely for their desired phytoremediation properties, both circumstances seldom repeated

in nature. Moreover, much of the biological pollutant abatement potential of the system exists through the synergistic activity of the entire community and, in purely direct terms, this largely means the indigenous microbes. Functionally, there are strong parallels between this and the processes of enhanced rhizospheric biodegradation described for terrestrial applications. While exactly the same mechanisms are available within the root zone in an aquatic setting, in addition, and particularly in the case of submerged vegetation, the surface of the plants themselves becomes a large extra substrate for the attached growth of closely associated bacteria and other microbial species. The combined rhizo- and circum-phyllo- spheres support a large total microbial biomass, with a distinctly different compositional character, which exhibits a high level of bioactivity, relative to other microbial communities. As with rhizodegradation on dry land, part of the reason is the increased localised oxygenation in their vicinity and the corresponding presence of significant quantities of plant metabolic exudates, which, as was mentioned in the relevant earlier section, represents a major proportion of the yearly photosynthetic output. In this way, the main role of the macrophytes themselves clearly is more of an indirect one, bringing about local environmental enhancement and optimisation for remediative microbes, rather than being directly implicated in activities of primary biodegradation. In addition, physico-chemical mechanisms are also at work. The iron plaques which form on the plant roots trap certain metals, notably arsenic (Otte, Kearns and Doyle 1995), while direct adsorption and chemical/biochemical reactions play a role in the removal of metals from the wastewater and their subsequent retention in sediments.

The ability of emergent macrophytes to transfer oxygen to their submerged portions is a well-appreciated phenomenon, which in nature enables them to cope with effective waterlogging and functional anoxia. As much as 60% of the oxygen transported to these parts of the plant can pass out into the rhizosphere, creating aerobic conditions for the thriving microbial community associated with the root zone, the leaf surfaces and the surrounding substrate. This accounts for a significant increase in the dissolved oxygen levels within the water generally and, most particularly, immediately adjacent to the macrophytes themselves.

The aerobic breakdown of carbon sources is facilitated by this oxygen transfer, for obvious reasons, and consequently it can be seen to have a major bearing on the rate of organic carbon biodegradation within the treatment system, since its adequate removal requires a minimum oxygen flux of one and a half times the input BOD loading. Importantly, this also makes possible the direct oxidation of hydrogen sulphide (H_2S) within the root zone and, in some cases, iron and manganese.

While from the earlier investigations mentioned on plant/metal interactions (Hutchinson 1975) their direct contribution to metal removal is small, fast-growing macrophytes have a high potential uptake rate of some commonly encountered effluent components. Some kinds of water hyacinth, *Eichhornia*

spp., for example, can increase their biomass by 10 g/m^2/day under optimum conditions, which represents an enormous demand for nitrogen and carbon from their environment. The direct uptake of nitrogen from water by these floating plants gives them an effective removal potential which approaches 6000 kg per hectare per year and this, coupled with their effectiveness in degrading phenols and in reducing copper, lead, mercury, nickel and zinc levels in effluents, explains their use in bioengineered treatment systems in warm climates.

Emergent macrophytes are also particularly efficient at removing and storing nitrogen in their roots, and some can do the same for phosphorus. However, the position of this latter contaminant in respect of phytotreatment in general is less straightforward. In a number of constructed wetland systems, though the overall efficacy in the reduction of BOD, and the removal of nitrogenous compounds and suspended solids has been high, the allied phosphorus components have been dealt with much less effectively. This may be of particular concern if phosphorus-rich effluents are to be routinely treated and there is a consequent risk of eutrophication resulting. It has been suggested that, while the reasons for this poor performance are not entirely understood, nor is it a universal finding for all applications of phytotreatment, it may be linked to low root zone oxygenation in slow-moving waters (Heathcote 2000). If this is indeed the case, then the preceding discussion on the oxygen pump effect of many emergent macrophytes has clear implications for biosystem design.

As has been established earlier, associated bacteria play a major part in aquatic plant treatment systems and microbial nitrification and denitrification processes are the major nitrogen-affecting mechanisms, with anaerobic denitrification, which typically takes place in the sediment, causing loss to atmosphere, while aerobic nitrification promotes and facilitates nitrogenous incorporation within the vegetation. For the effective final removal of assimilated effluent components, accessibly harvestable material is essential, and above water, standing biomass is ideal.

The link between the general desire for biodiversity conservation and the acceptability of created wetlands was mentioned earlier. One of the most important advantages of these systems is their potential to create habitats not just for 'popular' species, like waterfowl, but also for many less well-known organisms, which can be instrumental in bolstering the ecological integrity of the area. This may be of particular relevance in industrial or urban districts. At the same time, they can be ascetically pleasing, enhancing the landscape while performing their function. These systems can have relatively low capital costs, but inevitably every one must be heavily site specific, which means many aspects of the establishment financing are variable. However, the running costs are generally significantly lower than for comparable conventional treatment operations of similar capacity and efficacy. In part the reason for this is that once properly set up, a well-designed and constructed facility is almost entirely self-maintaining. However, the major contribution to low operational overheads comes from the system's low energy requirements, since gravity drives the water flow and all the

remediating organisms are ultimately solar powered, either directly or indirectly, via the photosynthetic action of the resident autotrophe community.

Aside of cost and amenity grounds, one major positive feature is that the effluent treatment itself is as good or better than that from conventional systems. When correctly designed, constructed, maintained and managed, plant-based treatment is a very efficient method of ameliorating wastewaters from a wide range of sources and in addition, is very tolerant of variance in organic loadings and effluent quality, which can cause problems for some of the alternative options. In addition, phyto-systems can often be very effective at odour reduction, which is often a major concern for the producers and processors of effluents rich in biodegradable substances.

Invariably, the better designed, the easier the treatment facility is to manage and in most cases, 'better' means simpler in practice, since this helps to keep the maintenance requirement to a minimum and makes maximum use of the existing topography and resources. Provision should also be made for climatic factors and most especially, for the possibility of flooding or drought. It is imperative that adequate consideration is given to the total water budget at the project planning stage. Although an obvious point, it is important to bear in mind that one of the major constraints on the use of aquatic systems is an adequate supply of water throughout the year. While ensuring this is seldom a problem for temperate lands, for some regions of the world it is a significant concern. Water budgeting is an attempt to model the total requirement, accounting for the net overall in- and outputs, together with the average steady-state volume resident within the system in operation. Thus, effluent inflow, supplementary 'clean' water and rainfall need to be balanced against off-take, evaporative and transpirational losses and the demands of the intended retention time required to treat the particular contaminant profile of a given wastewater. One apparent consideration in this process is the capacity of the facility. Determining the 'required' size for a treatment wetland is often complicated by uncertainty regarding the full range of wastewater volumes and component character likely to be encountered over the lifetime of the operation. The traditional response to this is to err on the side of caution and oversize, which, of course, has inevitable cost implications, but in addition, also affects the overall water budget. If the effluent character is known, or a sample can be obtained, its BOD can be found and it is then a relatively simple procedure to use this to calculate the necessary system size. However, this should only ever be taken as indicative. For one thing, bioengineered treatment systems typically have a lifespan of 15–20 years and the character of the effluent being treated may well change radically over this time, particularly in response to shifts in local industrial practice or profile. In addition, though BOD assessment is a useful point of reference, it is not a uniform indicator of the treatment requirements of all effluent components. For the bioamelioration process to proceed efficiently, a fairly constant water level is necessary. Although the importance of this in a drought scenario is self-evident, an unwanted influx of water can

be equally damaging, disturbing the established equilibrium of the wetland and pushing contaminants through the system before they can be adequately treated. Provision both to include sufficient supplementary supplies, and exclude surface water, is an essential part of the design process.

One aspect of system design which is not widely appreciated is the importance of providing a substrate with the right characteristics. A number of different materials have been used with varying degrees of success, including river sands, gravels, pulverised clinker, soils and even waste-derived composts, the final choice often being driven by issues of local availability. The main factors in determining the suitability of any given medium are its hydraulic permeability and absorbance potential for nutrients and pollutants. In the final analysis, the substrate must be able to provide an optimum growth medium for root development while also allowing for the uniform infiltration and through-flow of wastewater. A hydraulic permeability of between 10^{-3} and 10^{-4} m/s is generally accepted as ideal, since lower infiltration tends to lead to channelling and flow reduction, both of which severely restrict the efficiency of treatment. In addition, the chemical nature of the chosen material may have an immediate bearing on system efficacy. Soils with low inherent mineral content tend to encourage direct nutrient uptake to make good the deficiency, while highly humeric soils have been shown to have the opposite effect in some studies. The difficulties sometimes encountered in relation to phosphorus removal within wetland systems have been mentioned earlier. The character of the substrate medium can have an important influence on the uptake of this mineral, since the physico-chemical mechanisms responsible for its abstraction from wastewater in an aquatic treatment system relies on the presence of aluminium or iron within the rhizosphere. Obviously, soils with high relative content of these key metals will be more effective at removing the phosphate component from effluent, while clay-rich substrates tend to be better suited to lowering heavy metal content.

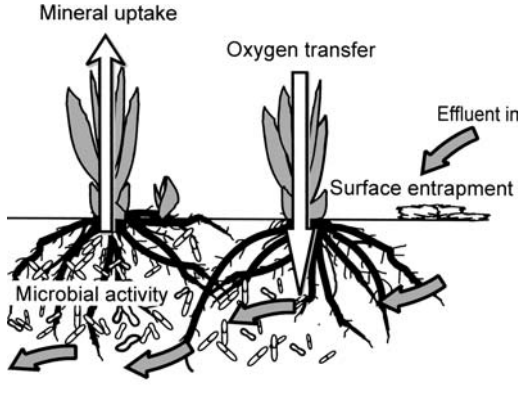

Figure 7.3 *Diagrammatic root zone activity*

Engineered reed beds are probably the most familiar of all macrophyte treatment systems, with several high profile installations in various parts of the globe having made the technology very widely accessible and well appreciated. This approach has been successfully applied to a wide variety of industrial effluents, in many different climatic conditions and has currently been enjoying considerable interest as a 'green' alternative to septic tanks for houses not joined up to

Table 7.1 *Typical analysis for leachate treatment by reed-bed systems*

Component	Inlet conc.	Outlet Conc.	% reduction
Chlorobenzene	83.0	<1.0	>98.7
1,2-Dichlorobenzene	2.0	<1.0	>50
1,3-Dichlorobenzene	3.6	<1.0	>72
1,4-Dichlorobenzene	9.8	<1.0	>89
1,2,4-Trichlorobenzene	0.22	<0.05	>77
α-hydrocarbons	0.11	0.037	>66
β-hydrocarbons	0.22	<0.005	>97.7
γ-hydrocarbons	0.45	0.026	>94
δ-hydrocarbons	1.0	0.21	>79
ε-hydrocarbons	1.3	0.31	>76
2-Chlorophenol	3.7	0.84	>77
3,4-Chlorophenol	0.63	<0.5	>92
2,5-Dichlorophenol	5.0	0.56	>88
3,4-Dichlorophenol	4.9	<0.5	>98.9
3,5-Dichlorophenol	0.93	<0.5	>94
2,3,4,6-Tetrachlorophenol	6.4	<1.0	>84
Pentachlorophenol	2.7	<1.0	>62
2,2′,5,5′-Penta-PCB	0.11	<0.05	>54
2,2′,4,4′,5,5′-Hexa-PCB	0.046	<0.025	>45
Naphthalene	17.0	<0.05	>99.7
1-Methylnapthalene	1.7	<0.05	>97
2-Methylnapthalene	0.58	<0.05	>91
Acenapthalene	2.2	<0.05	>97
Fluorene	3.0	<0.05	>98
Phenanthrene	2.7	<0.05	>98
Anthracene	0.4	<0.05	>87
Pyrene	0.29	<0.05	>82
Fluoranthene	0.5	<0.05	>90
Trichloroethene	1.8	<0.05	>97
Tetrachloroethene	3.1	0.11	>96
Benzene	52.0	<1.0	>98
Toluene	1.8	<1.0	>44
Ethylbenzene	16.0	<1.0	>93
m-/p- Xylene	4.1	<1.0	>75
o-Xylene	3.1	<1.0	>67
1,2,4-Trimethylbenzene	3.1	<1.0	>67

Concentrations are expressed in micrograms per litre.
After Kickuth.

mains sewerage. At its heart is the ability of reeds, often established as monocultures of individual species, or sometimes as oligocultures of a few, closely related forms, to force oxygen down into the rhizosphere, as has been previously discussed. Many examples feature *Phragmites* or *Typha* species, which appear to be particularly good exponents of the oxygen pump, while simultaneously able to support a healthy rhizospheric microfloral complement and provide a stable root zone lattice for associated bacterial growth and physico-chemical processing of rhizo-contiguous contaminants. Isolated from the surrounding ground by an impermeable clay or polymer layer, the reed bed is almost the archetypal emergent macrophyte treatment system.

The mechanisms of action are shown in Figure 7.3 and may be categorised as surface entrapment of any solids or relatively large particulates on the growing medium or upper root surface. The hydraulic flow draws the effluent down through the rhizosphere, where the biodegradable components come into direct contact with the root zone's indigenous micro-organisms, which are stimulated to enhanced metabolic activity by the elevated aeration and greater nutrient availability. There is a net movement of oxygen down through the plant and a corresponding take-up by the reeds of nitrates and minerals made accessible by the action of nitrifying and other bacteria.

These systems are very efficient at contamination removal, typically achieving 95% or better remediation of a wide variety of pollutant substances, as demonstrated in Table 7.1, which shows illustrative data on the amelioration of landfill leachates by this system.

Nevertheless, reed beds and root zone treatment techniques in general are not immune from a range of characteristic potential operational problems, which can act to limit the efficacy of the process. Thus, excessive waterlogging, surface runoff, poor or irregular substrate penetration and the development of preferential drainage channels across the beds may all contribute to a lessening of the system's performance, in varying degrees.

Nutrient Film Techniques (NFT)

An alternative approach to the use of aquatic macrophytes, which was tried experimentally, involved growing plants on an impermeable containment layer, in a thin film of water. In this system, the wastewater flowed directly over the root mass, thereby avoiding some of the mass transfer problems sometimes encountered by other aquatic phytotreatment regimes. Though the early work indicated that it had considerable potential for use in the biological treatment of sewage and other nutrient-rich effluents, it does not appear to have been developed further and little is known as to the conditions which govern its successful practical application. One interesting aspect which did, however, emerge was that the cultivation system could also be extended to most terrestrial plants, which may yet be of possible relevance to the future development of land-based phytotreatments.

Algal Treatment Systems (ATS)

Algae have principally been employed to remove nitrogen and phosphorus from wastewaters, though some organic chemicals can also be treated and a relatively new application has emerged which makes use of their efficient carbon sequestration potential.

Effluent treatment

Algal effluent treatment systems work on the basis of functional eutrophication and rely on a dynamic equilibrium between the autotrophic algae themselves and the resident heterotophic bacteria, which establishes a two-stage biodegradation/assimilation process, as shown in Figure 7.4. In effect this is an ecological microcosm in which organic contaminants present in the wastewater are biologically decomposed by the aerobic bacteria, which make use of oxygen provided by algal photosynthesis, while the algae grow using the nutrients produced by this bacterial breakdown, and photosynthesise producing more oxygen.

Though the process is self-sustaining, it is also self-limiting and left to proceed unchecked, will result in the well-appreciated characteristic eutrophic stages leading to the eventual death of all component organisms, since true climactic balance is never achieved in the presence of continuously high additional nutrient inputs. The removal of excess algal and bacterial biomass is, therefore, an essential feature, vital to maintaining the system's efficiency.

Of all the engineered algal systems for effluent, the high rate algal pond (HRAP) is one of the most efficient and represents a good illustration of this use of phytotechnology. Figure 7.5 shows a typical example.

The system consists of a bioreactor cell in the form of a relatively shallow reservoir, typically between 0.2–0.6 metres deep, with a length to width ratio of 2:1 or more, the idea being to produce a large surface area to volume ratio. The void is divided with internal baffles forming walls, to create a channel through which the effluent flows. A mechanically driven paddle at the end nearest to the effluent input both aerates and drives the wastewater around the system.

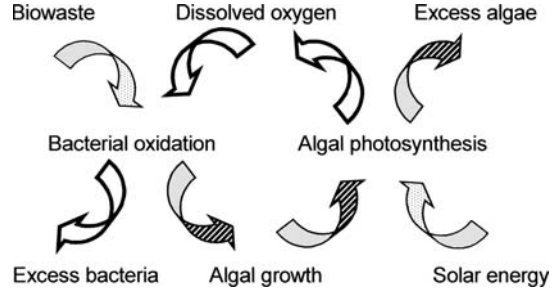

Figure 7.4 *Algal and bacterial equilibrium*

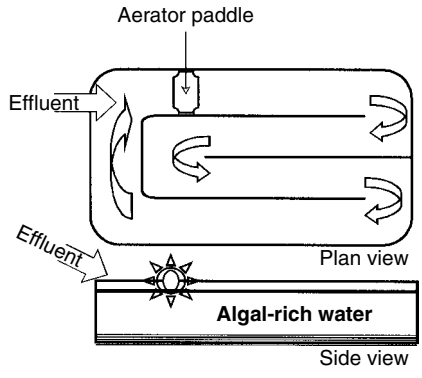

Figure 7.5 *High rate algal pond*

These ponds are not sensitive to fluctuations in daily feed, either in terms of quantity or quality of effluent, providing that it is fundamentally of a kind suitable for this type of treatment. Consequently, they may be fed on a continuous or intermittent basis. The main influences which affect the system's performance are the composition of the effluent, the efficiency of mixing, the retention time, the availability and intensity of light, pond depth and temperature. The latter two factors are particularly interesting since they form logical constraints on the two groups of organisms responsible for the system's function, by affecting the autotrophe's ability to photosynthesise and the heterotrophe's to respire. While a deeper cell permits greater resident biomass, thus elevating the numbers of micro-organisms available to work on the effluent, beyond a certain limit, the law of diminishing returns applies in respect of light available to algae in the lower reaches. Warmer temperatures increase metabolic activity, at least within reason, and the rate of straightforward chemical reactions doubles per 10 °C rise, but at the same time, elevated water temperatures have a reduced oxygen-carrying capacity which affects the bacterial side of the equilibrium mentioned earlier. As with so much of environmental biotechnology, a delicate balancing act is required.

After a suitable retention period, which again depends on the character of the effluent, the design and efficacy of the treatment pond and the level of clean-up required, the water is discharged for use or returned to watercourses. Obviously, after a number of cycles, algal and bacterial growth in a functionally eutrophic environment would, as discussed earlier in the section, begin to inhibit, and then eventually arrest, the biotreatment process. By harvesting the algal biomass, not only are the contaminants, which to this point have been merely biologically isolated, physically removed from the system, but also a local population depression is created, triggering renewed growth and thus optimised pollutant uptake. The biomass recovered in this way has a variety of possible uses, of which composting for ultimate nutrient reclamation is without doubt the most popular, though

various attempts have also been made to turn the algal crop into a number of different products, including animal feed and insulating material.

Carbon sequestration

Their use as a carbon sink is a simpler process, only requiring the algae themselves. However, even as a functional algal monoculture, just as with the joint algal/bacterial bioprocessing for effluents, without external intervention to limit the standing burden of biomass within the bioreactor, reduced efficiency and, ultimately, system collapse is inevitable.

In nature, huge amounts of many elements are held in global reservoirs, regulated by biogeochemical cycles, driven by various interrelated biological and chemical systems. For carbon, a considerable mass is held in organic and inorganic oceanic stores, with the seas themselves being dynamic and important component parts of the planetary carbon cycle. Marine phytoplankton utilise carbon dissolved in the water during photosynthesis, incorporating it into biomass and simultaneously increasing the inflow gradient from the atmosphere. When these organisms die, they sink, locking up this transient carbon and taking it out of the upper oceanic 'fast' cycle into the 'slow' cycle, which is bounded by long-term activities within the deep ocean sediments. In this respect, the system may be likened to a biological sequestration pump, effectively removing atmospheric CO_2 from circulation within the biosphere on an extended basis. The number, mass and extent of phytoplankton throughout the world's seas thus provide a carbon-buffering capacity on a truly enormous scale, the full size of which has only really become apparent within the last 10–15 years, with the benefit of satellite observation.

In the century since its effectiveness as a means of trapping heat in the atmosphere was first demonstrated by the Swedish scientist, Svante Arrhenius, the importance of reducing the global carbon dioxide emissions has come to be widely appreciated. The increasing quantities of coal, oil and gas that are burnt for energy has led to CO_2 emissions worldwide becoming more than 10 times higher than they were in 1900 and there is over 30% more CO_2 in the air, currently around 370 parts per million (ppm), than before the Industrial Revolution. Carbon dioxide is responsible for over 80% of global warming and according to analysis of samples of the Antarctic ice, the world today has higher levels of greenhouse gases than at any time in the past 400 000 years. The UN Intergovernmental Panel on Climate Change has warned that immediate action is required to prevent further atmospheric increases above today's level. In the absence of swift and effective measures to control the situation, by 2100 they predict that carbon dioxide concentrations will rise to 550 ppm on the basis of their lowest emission model, or over 830 ppm in the highest.

In 1990, over 95% of the western industrialised nations' emissions resulted from burning fossil fuels for energy, with the 25% of the world's population who live in these countries consuming nearly 80% of the energy produced globally.

Unsurprisingly, energy industries account for the greatest share (36%) of carbon dioxide emissions, a large 1000 Megawatt coal-fired power station releases something in the region of $5\frac{1}{2}$ million tonnes of CO_2 annually. Clearly, the current focus on reducing fossil fuel usage, and on minimising the emissions of carbon dioxide to atmosphere, is important. In one sense, the most straightforward solution to the problem is simply to stop using fossil fuels altogether. However, this is a rather simplistic view and just too impractical. While great advances have been made in the field of renewable energy, a wholesale substitution for gas, coal and oil is not possible at this time if energy usage is to continue at an unabated rate. The potential role of existing nonfossil fuel technology to bridge the gap between the current status quo and a future time, when renewables meet the needs of mankind, is a vital one. However, it is ridiculous to pretend that this can be achieved overnight, unless the 'global village' really is to consist of just so many mud huts.

In many respects, here is another case where, if we cannot do the most good, then perhaps we must settle for doing the least harm and the application of phytotechnology stands as one very promising means by which to achieve this goal. The natural contribution of algal photosynthesis to carbon sequestration has already been alluded to and the use of these organisms in an engineered system to reduce CO_2 releases, simply capitalises on this same inherent potential in an unaltered way.

There have been attempts to commercialise the benefits of algae as carbon sinks. In the early 1990s, two prototype systems were developed in the UK, aimed at the reduction of CO_2 emissions from various forms of existing combustion processes. The BioCoil was a particularly interesting integrated approach, removing carbon dioxide from generator emissions and deriving an alternative fuel source in the process. The process centred on the use of unicellular algal species in a narrow, water-containing, spiral tube made of translucent polymer, through which the exhaust gases from the generator was passed. The carbon dioxide rich waters provided the resident algal with optimised conditions for photosynthesis which were further enhanced by the use of additional artificial light. The algal biomass recovered from the BioCoil reactor was dried, and being unicellular, the effective individual particle size tended to the dimensions of diesel injection droplets, which, coupled with an energy value roughly equivalent to medium grade bituminous coal at 25 MJ/kg, makes it ideal for use in a suitable engine without further modification. Despite early interest, the system does not appear to have been commercially adopted or developed further.

Around the same time, another method was also suggested by one of the authors. In this case, it was his intent specifically to deal with the carbon dioxide produced when biogas, made either at landfill sites or anaerobic digestion plants, was flared or used for electricity generation. Termed the algal cultivation system and carbon sink (ACSACS), it used filamentous algae, growing as attached biofilter elements on a polymeric lattice support. CO_2 rich exhaust gas

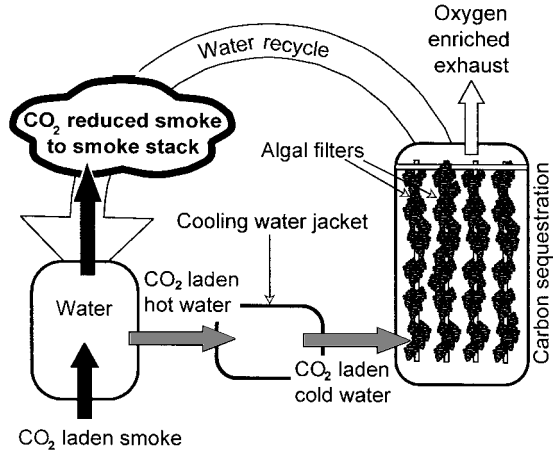

Figure 7.6 *Schematic ACSACS*

was passed into the bottom of a bioreactor vessel, containing the plastic filter elements in water, and allowed to bubble up to the surface through the algal strands as shown in Figure 7.6.

Again, this approach to carbon sequestration was based on enhanced intra-reactor photosynthesis, the excess algal biomass being harvested to ensure the ongoing viability of the system, with the intention of linking it into a composting operation to achieve the long-term carbon lock-up desired. The ACSACS though performing well at both bench and small pilot scale, never attained industrial adoption though remaining an interesting possible adjunct to the increasing demand for methane flaring or utilisation at landfills.

A similar idea emerged again recently, with a system being developed by Ohio University, which, in a perfect example of selecting an organism from an extreme environment to match the demands of a particular manmade situation, utilises thermophilic algae from hot springs in Yellowstone National Park. In this process, which has received a $1 million grant from the US Department of Energy, smoke from power stations is diverted through water to permit some of the CO_2 to be absorbed and the hot, carbonated water produced then flows through an algal filter formed on vertical nylon screens.

This design, which is essentially similar to the earlier ACSACS, enables the largest possible algal population per unit volume to be packed into the filter unit, though like the previously described HRAP, light is a limiting factor, since direct sunlight will only penetrate through a few feet of such an arrangement. However, it is claimed that these carbon biofilters could remove up to 20% of the carbon dioxide, which would, of course, otherwise be released to atmosphere. This makes solving the problem something of a priority. One solution involves the use of a centralised light collector, connected to a series of fibre-optic cables

linked to diffusers within the vessels to provide adequate illumination within the filters. An alternative approach has been put forward, using large artificial lakes, but this would require a much larger land bank to produce the same effect, since they would have to be very shallow by comparison. It has also been suggested that cooling the carbonated water first, a feature of both the BioCoil and ACSACS, would allow normal mesophilic algae to be used, which take up CO_2 more efficiently. Whether this technique will prove any more successful in gaining industrial or commercial acceptance than either of the earlier British systems remains to be seen.

Pollution Detection

One final, and currently emerging, application of phytotechnology to the environmental context involves the possible use of plants in a variety of industrial sectors as pollution detectors. The aim is principally to provide valuable information about the toxicological components of contamination from a wide variety of sources, including the automotive, chemical and textile industries. Unlike biosensors, which tend to be designed around isolated biochemical reactions, in this approach, the plants are used as entire biological test systems. Moreover, unlike conventional chemical analytical methods which produce quantifiable, numeric measurements, the varieties used have been selected for their abilities to identify contaminants by reacting to the specific effects these substances have on the plant's vital functions. Thus, by directing the focus firmly onto the obvious and discernible biological consequences of the pollutants and then codifying this into a diagnostic tool, the assessment process is made more readily available to a wider range of those who have an interest in pollution control.

The development of this technology is still in the initial stages, but it would appear to open up the way for a controllable method to determine pollutant effects. It seems likely that they will be of particular value as early detection systems in the field, since they are functional within a broad range of pH and under varied climatic conditions. An additional benefit is that they are responsive to both long-term pollution or incidental spillages and can be applied to either laboratory or on-site investigations to monitor air, soil or water, even on turbid or coloured samples, which often cause anomalous readings with spectrophotometric test methods.

Closing Remarks

The scope of opportunities available to phytotechnological interventions are, then, wide ranging, though presently, much of this potential remains unexplored. It

seems likely that the increasingly prioritised initiatives to find low-cost systems to bring about effective remediation, effluent control and carbon sequestration will begin to reverse this situation in the near future. However, it is unavoidably true that the wider uptake of all plant-utilising bioengineering applications will depend as much on local modalities as on the actual state of the biotechnologies themselves. In this respect, phytotechnology has much in its favour. For one thing, it has the enormous benefit of virtually assured universal public acceptance, which is rare for any biotechnology. Commercially, it is a relatively low-intervention, highly 'green' and thoroughly noncontentious approach to environmental management, which has a strong potentially positive contribution to corporate image, with a relatively low negative influence on the balance sheet. The great advantage that almost all plant-based systems bring to biological engineering is the tremendous energy saving represented by their solar-powered nature. This, combined with their essentially integrated and intrinsically complex array of metabolic mechanisms makes a variety of plant species extremely useful in an environmental context, and typically without any additional need for modification. Given their inherent flexibility, acceptability, efficiency and cost-effectiveness, it is difficult to imagine that phytotechnological systems will not be further developed and more widely adopted in the future.

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Case Study 7.1 Naturalising Phytoremediators (Dyfed and Powys, Wales)

Though necessary for swift clean-up operations where time is limited or the environmental risk high, not all examples of the removal of industrial contamination by biological means involve highly engineered interventions. Many of the most effective means are those which work with nature.

The combined effects of natural attenuation, phytotechnology and selective pressures in remediation can be seen at a number of sites throughout Mid-Wales. Earlier mining of the area's rich mineral deposits, especially silver and lead, inevitably brought about significant disturbances to the immediate environment. In some places, quantities of the relatively metal-enriched 'spoil' generated remain as heaps, while elsewhere it has been spread over the original ground surface in an attempt to reduce the effects of localised accumulation. While the low concentration, in absolute terms, makes extraction and refining commercially uneconomic, the level of contamination is sufficient to pose a challenge to plant growth.

Today, these once barren spoil sites have, in the main, been revegetated and only a small number remain bare, in areas of the highest residual metal concentration, as the actions of soil microbes and indigenous plants over the intervening years, have contributed to a gradual reduction in contamination. Laboratory investigations of the colonising plant species have revealed high innate tolerances of relatively elevated metal concentrations, representing an example of selective advantage in response to environmental pressure. Consequently, particular strains of grasses and other vegetation have developed which are able to thrive under these conditions, which has considerably improved the visual appearance of these old sites. In addition, a number act as natural phytoremediators, which further assists the wider amelioration process.

Case Study 7.2 Engineered Phytoremediation (Billingham, England)

One of the best examples of industrial-scale use of reed beds is the one managed by Air Products (Chemicals) Teeside Ltd on the old ICI site in Billingham, England where *Phragmites* are used to clean the effluent from the methylmethacrylate plant prior to discharge into the river Tees. This wastewater principally contains phenol, methanol, acetone, chlorinated hydrocarbons and amines.

Although modifying the production process to eliminate much of the problem would have been possible for a new facility, it was too costly to achieve retrospectively and so a variety of biological treatments were explored to ameliorate the effluent. Although the cheapest capital option was an oxygen activated sludge system, the phytotechnology approach was eventually selected since its running costs were very low and, additionally, because sludge disposal was not required.

One of the keys to effective plant-based intervention is ensuring that the right plant is chosen. It was particularly relevant in this case, since several different *Phragmites* reeds are used in this role around the world. Six varieties were tested for effluent survival under local environmental conditions. On the basis of this work, three were selected for use in the pilot trials–Alexander reed from Germany and strains from two sites in the area, namely Billingham Golf Course and Salt Home Farm.

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Seven beds, amounting to a total of five hectares (12.5 acres) were constructed in 1990, with the design specification of ultimately being able to treat 3000 m^3 of effluent daily.

The system has some noteworthy and unusual features. Reed beds are often constructed by excavation, but at the Billingham site, one-metre high clay bunds were created surrounding each of the seven individual beds. The resulting cells were lined with an impermeable polymer membrane, equipped with drainage pipes and filled in with soil before planting. The drainage arrangements are also an important part of the overall process. Unlike many macrophyte beds, where the main prevailing hydraulic conduction lies effectively in a horizontal direction, at Billingham much of the treatment takes place during the vertical flow, down through the soil, which is further enhanced by the provision of outlet pipes at intervals across the bed, rather than simply at one end. As a result of this somewhat atypical design, it has been possible to achieve the required level of effluent polishing with a significantly smaller land requirement than would otherwise have been necessary, which has important practical and economic implications.

Historically, the environmental record of the chemical industry globally has not always been a glorious one. However, the successes of commercial projects like this one, clearly show that the application of biotechnology in general, and phytotechnology in particular, can bring very real benefits, without compromising either the local ecology or economy.

8

Biotechnology and Waste

As mentioned in the first chapter, waste represents one of the three key intervention points for the potential use of environmental biotechnology. Moreover, in many ways this particular area of application epitomises much of the whole field, since the management of waste is fundamentally unglamorous, typically funded on a distinctly limited budget and has traditionally been viewed as a necessary inconvenience. However, as the price of customary disposal or treatment options has risen, and ever more stringent legislation been imposed, alternative technologies have become increasingly attractive in the light of their greater relative cost-effectiveness. Nowhere has this shift of emphasis been more apparent than in the sphere of biological waste treatment.

With all of environmental biotechnology it is a self-evident truism that whatever is to be treated must be susceptible to biological action and hence the word 'biowaste' has been coined to distinguish the generic forms of organic-origin refuse which meet this criterion, from waste in the wider sense, which does not. This approach also removes much of the confusion which has, historically, dogged the issue, since the material has been variously labelled *putrescible, green, yard, food* or even just *organic* waste, at certain times and by differing authors, over the years. By accepting the single term *biowaste* to cover all such refuse, the difficulties produced by regionally, or nationally, accepted criteria for waste categorisation are largely obviated and the material can be viewed purely in terms of its ease of biodegradability. Hence a more process-based perspective emerges, which is often of considerably greater relevance to the practical concerns of actually utilising biotechnology than a straightforward consideration of the particular origins of the waste itself.

The Nature of Biowaste

Biowaste arises from a number of human activities, including agriculture, horticulture and industry, broadly falling into one of the following three major categories: faeces/manures, raw plant matter or process waste. This fits neatly into the process-orientated approach mentioned above, since the general characteristics of each are such that biological breakdown proceeds in essentially the same manner within the group and, thus, the ease of their decomposition is closely similar. Although, at least chemically speaking, biowaste can be seen

as being characterised by a high carbon content, this definition is so wide as to include the vast majority of the substances for which all environmental biotechnologies are viable process options. Hence, in the present discussion, biowaste is limited to substances which have been derived from recently living matter, with the approaches available to deal with other carbon-rich materials having already been examined in the preceding chapters on pollution control, contaminated land and effluent treatment.

Composition of biowaste

Biowaste of animal origin such as that contained in sewage and soiled animal bedding contains unabsorbed fats, proteins and carbohydrates, resulting from incomplete digestion of ingested food of animal and plant origin. In addition, abattoir waste would include all of the above and a substantial proportion of fats and protein, derived from the slaughtered animal. In addition, materials excreted by the animal include metabolic breakdown products such as urea and other small nitrogen-containing materials, for example partially degraded bile pigments. Live and dead bacteria, normally resident in animal gut are also present in the biowaste and so contribute their own fats, proteins, carbohydrates and nucleic acids.

In addition to all the components listed above, biowaste of plant origin will contain cellulose, hemicelluloses and lignin. Cellulose is worthy of note given that estimates of over 50% of the total organic carbon in this biosphere is to be found in the form of cellulose. This is unsurprising, since wood is approximately 50% cellulose and cotton is almost 100% cellulose. This macromolecule is an unbranched polysaccharide comprising D-glucose units linked by β 1–4 linkages (see Figure 2.3). It is this β link, rather than the α link found in its animal equivalent, glycogen, which prevents cellulose being broken down by the metabolic pathways in animals. The initial step in the degradation of cellulose is the removal of a glucose molecule from one end of the long chain which is a reaction catalysed by the enzyme *cellulase.* Where cellulose is degraded in animals it is by bacteria resident in the animal rumen or gut, which possess *cellulase*. There are also many bacteria living outside the gut, both aerobes and soil anaerobes (Monserrate, Leschine and Canale-Parola 2001) responsible for cellulose metabolism. Another major constituent of plant material, the hemicelluloses, are also polysaccharides but the subunit in this case is the five-carbon sugar D-xylose, also joined 'head to tail' by a β 1–4 linkage. Otherwise, hemicellulose is not related to cellulose despite the similar name. Unlike cellulose which comprises only D-glucose and in an unbranched structure, the family of hemicelluloses has side chains and these may comprise any of a variety of sugars one of which may be the five-carbon sugar, arabinose. The function of hemicelluloses in plants is to form part of the matrix which holds the cellulose fibrils together to improve strength and rigidity of the plant tissue. Lignin is also a very abundant material in plants and is estimated to comprise almost 25% of the dry weight of wood. Totally unlike cellulose or hemicelluloses, which are polymers

of sugars and therefore are carbohydrates, lignin is a polymer of the two amino acids, phenylalanine and tyrosine. Despite its abundance, its structure is poorly understood, in part a tribute to the fact that it is extremely resistant to degradation and therefore presents problems to the analyst. Fortunately for the natural process of carbon and nitrogen recycling on which our biosphere depends, fungi degrade lignin and, in addition, some microbes, like those resident in the gut of termites can perform the same function.

Biowaste makes up a huge percentage of refuse; some 2500 million tonnes arise each year in the European Union alone (Lemmes 1998) and this is a figure which many authorities suggest increases by between 3–5% annually. Although the focus of much of this chapter is firmly centred on the biowaste component of municipal solid waste (MSW), since this is the kind of waste which most directly concerns the largest number of people, it is important to be aware that this does not represent the full picture, by any means. Of these 2500 million tonnes of biowaste, 1000 million is agricultural in origin, 550 million tonnes consists of garden and forestry waste, 500 million is sewage and 250 million results from the food-processing industry, leaving MSW only to make up the remaining 200 million tonnes. The scale of the problem is large, one study suggesting that an annual total of between 850–1000 kg (total solids) of material suitable for biological treatment are produced per person (Frostell 1992). There is general agreement that biowaste accounts for around a third of the industrialised world's municipal waste stream and that a further 30% or so is also expressly biodegradable, such a definition including paper. In the light of this, the fact that the potential for the development and application of approaches based on biological processing has not yet been more rigorously or comprehensively explored remains somewhat surprising. Moreover, with society in general increasingly committed to the 'green' ideals of maximised recycling and the rational utilisation of waste, it is difficult to see how such goals can realistically ever be expected to be met, without significant attention being paid to the biowaste issue. In this respect, the writing may already be on the wall, since the demands of legislation appearing in Europe, the USA and elsewhere has begun to drive fundamental reappraisals of the way in which all refuse is regarded. In particular, regulatory changes designed to reduce the amount of raw biodegradable material destined for landfill must ultimately come to promote biotechnologies which can treat this material in an effective and more environmentally acceptable way. While predicting the future is, of course, notoriously difficult, it seems likely that biological processing will assume a more central role in future waste management regimes, which presents both exciting possibilities and some genuine challenges to the industry itself. However, in order to understand why, it is important to consider the current difficulties posed by biowaste under traditional disposal routes.

Although a number of changes in the whole perception of waste have led to a variety of relatively new options receiving attention, generally throughout the world, the vast majority of refuse is dealt with either by means of landfill or

Table 8.1 *A comparison of selected national waste management arrangements, recycling rates and MSW biowaste component*

Country	Landfill (%)	Incineration (%)	Recycled (%)	Biowaste (%)
Austria	65	11	24	27
Belgium	43	54	3	47
Canada	67	4	29	34
Denmark	20	55	25	37
Finland	66	4	30	33
France	59	33	8	30
Germany	46	36	18	30
Netherlands	30	42	28	35
Japan	21	74	5	26
Norway	68	18	14	35
Sweden	34	47	19	38
Switzerland	11	47	42	30
UK	85	9	6	30
USA	61	15	24	32

Sources: IEA Bioenergy, European Commission & relevant Embassies.

incineration. Different countries and administrations have favoured one or the other at various times and, as with all things to do with waste, local custom and circumstance have played a major part in shaping the current status quo. While it is beyond the scope of the present discussion to examine this in any depth, Table 8.1 may help to provide some indication of the wider situation.

Although there has been considerable development in incineration technology over the years and today's facilities, with their energy recovery, power generation and district heating potential, are a far cry from the simple smoking stacks of old, for biological origin waste, mass burn incinerators cannot be viewed as the ideal solution. Hence, while the incineration v. landfill argument still rages, and has been revisited with renewed vigour in some circles in the light of the implications of recent European legislation on landfill, the fact remains that, at least from the standpoint of biowaste, both are nothing more than disposal routes. Significant amounts of wet organic material, which is itself largely composed of water to begin with, may be an inconvenience to the incinerator operator; the situation in landfill is worse.

Landfill

Left to its own devices, all discarded biological waste gradually undergoes a natural process of biodegradation, typically beginning with autolysis and culminating in putrefaction. The speed at which this progresses is governed by a number of factors such as the nature and freshness of the material, the temperature, moisture and so on. When this happens in the open air, or in the upper levels of the soil, decomposition is aerobic, the organic material being mineralised and carbon dioxide (CO_2) released as the major gaseous product. However, though

biowaste awaiting collection in dustbins and even, to some extent, when only recently delivered to landfill, initially begins to break down in this way, older putrescible material, buried deeper, experiences conditions effectively starved of oxygen. In this environment, the degradation process is anaerobic and mineralisation continues with broadly equal amounts of methane (CH_4) and carbon dioxide being produced. This resultant mix is known as landfill gas and typically contains a number of trace gases of varying chemical composition. At the functional level, the mechanism of this reaction is very complex, with hundreds of intermediary reactions and products potentially involved and many requiring additional synergistic substances, enzymes or other catalysts. Methanogenesis is discussed more fully elsewhere, but it is possible to simplify the overall process thus:

$$\text{Organic material} \longrightarrow CH_4 + CO_2 + H_2 + NH_3 + H_2S$$

The production of methane is a particular worry in environmental terms since, although there is some disagreement as to the exact figure, it is widely accepted as more than 30 times more damaging as a greenhouse gas than a similar amount of carbon dioxide. It was precisely because of these concerns that the European Union began its drive to produce statutory controls on the amount of biodegradable material permitted to be disposed of by this route. Without going into lengthy descriptions of the final legislation adopted, or the history of its stormy 10-year passage into European law, it is fair to say that the elements of the Landfill Directive which relate to biowaste require considerable changes to be made in waste management practice. This is of particular importance for those countries, like the UK, with a previously heavy traditional reliance on this method. A series of stepped major reductions in the amount of material entering landfill are required and a timetable has been imposed for their implementation. By 2020 at the latest, all EU member states must have reduced their biowaste input into landfill by 65% of the comparable figure for 1995. According to the Directive, 'biodegradable' is expressly defined as any 'waste that is capable of undergoing anaerobic or aerobic decomposition, such as food and garden waste, and *paper and paperboard*' (DETR 1999a). This has particular implications for currently landfill-dependent nations. The most recent Environment Agency figures show that 32% (by dry weight) of MSW production in the UK is paper. This represents its single largest biodegradable component, using the Directive definition, pushing the traditional biowaste element into second place by 11% (DETR 1999a). Taking into account the additional contributions of 1% textiles, 3.5% 'fines', 4% miscellaneous combustibles and noncombustibles at 1%, the grand total of 'biodegradable' inclusions in the UK waste stream comes to 62.5%, based on figures from this same study (DETR 1999a). Making up more than half of the total on its own, paper is, then, of great potential importance, and it is clear that no attempt at reaching the levels of reduction demanded by the new law can afford to ignore this material.

The question of methane production, so central to the original thrust of the legislation has been addressed by requiring sites to collect the landfill gas produced and use it for energy generation, conceding that it may be flared off where for some reason this is not possible.

A second potential environmental problem typically associated with landfills is the production of polluting leachates, which can be aggravated by the dumping of biowaste. Water percolating through the site tends to leach out both organic and inorganic substances which can lead to contamination of the groundwater. The persistence of pathogens and the potential translocation of many biologically active chemicals have recently become of increasing concern in the light of growing (though largely circumstantial) evidence of health problems associated with proximity to certain landfill sites. However, there is considerable variance between many aspects of different facilities and, additionally, much uncertainty as to the extent of any possible exposure to chemicals found therein (Vrijheid 2000). The UK government commissioned the world's most extensive study to date into the potential health risks of living within 2 kilometres of landfills, to examine the incidence of low birth weight, congenital defects, stillbirths and cancers in the vicinity of 9565 landfill sites, with a sample size in excess of some 8 million pregnancies. This revealed a 7% increase in the rate of both chromosomal and nonchromosomal birth defects (Elliott *et al*. 2001) but the expert advisory committee observed that this represented only a small excess risk and might well be accounted for by factors other than those directly attributable to landfill itself. While domestic landfill operations, then, may well be of little significant threat to those around them, the situation for hazardous waste sites, though admittedly less well investigated, appears somewhat different.

The findings of the recent, new investigation (Vrijheid *et al*. 2002) of data originating from a smaller study of certain European landfills which accept hazardous waste (Dolk *et al*. 1998) suggests a 40% increase in chromosomal birth defects and a 33% increase in the risk of nonchromosomal abnormalities, within a 3 kilometre radius. However, whether the observed increase in risk arises merely from living near such a hazardous waste site, or as a result of other factors as yet unknown, remains unclear. Greater understanding of the true scope of landfill releases, their potential toxicity and the possible exposure pathways will be required to permit more meaningful interpretations of the epidemiological data to be made.

Even where there is nothing to suggest an adverse effect on the local population, high concentrations of biowaste-derived leachate remain undesirable. Such rich liquors provide heterotrophic micro-organisms with a ready and abundant source of food. In conditions of relatively low organic loading, a dynamic equilibrium is reached between the bacteria breaking this material down and the autotrophic organisms, typically algae, which subsequently make use of these breakdown products. The oxygen balance works, since the requirements of the aerobic decomposers is offset by the contribution of the photosynthetic algae

present. However, under conditions of high organic loading, the oxygen demand of the bacteria exceeds the carrying capacity of the water and the algae's ability to replenish it. Hence a downward spiral develops, which ultimate leads to locally anaerobic conditions.

Although 'waste' is itself one of the three key potential intervention points for environmental biotechnology, it should be clear from the preceding discussion that there is considerable capacity for biological waste treatment technologies to contribute heavily to another, namely the reduction of pollution. To try to set this in context, it is quite common for landfill leachate analysis ranges to be quoted based on the average values obtained from a number of established sites. However, this can lead to a significant distortion of the true picture since, particularly for newer landfills (where the biochemical activity tends more to early acetogenic fermentation than 'old' post-methanogenic or even semi-aerobic processes) a degree of under-representation often occurs for some substances. For example, 'young', acetogenic leachate is typically below pH 7 and of high COD, though much of the latter is biodegradable. The bacteria responsible for the biological breakdown at this point in the site's life may be anaerobic, aerobic or facultative anaerobes. In older landfills, methanogenic bacteria predominate, which are strict anaerobes and can only assume and maintain their dominant position in the absence of oxygen. Such conditions develop in time as the normal sequence of events involves the early acetogenic bacteria gradually using up the available oxygen and producing both the necessary anaerobic environment and acetate as a ready food source for the methanogens which follow in succession, as the site ages.

The full picture of the pollution potential of landfill leachate is more complex than might at first be supposed, if for no other reason than, though it is spoken of as if it were a single commodity, leachate is a highly variable and distinctly heterogeneous substance. It is influenced by the age, contents and management of the landfill of its origin, as well as by the temperature and rainfall of the site. Moreover, all of these factors interact and may vary considerably, even in the relatively short term, not to mention over the decades of a typical landfill's lifetime. The general range of values for landfill leachate established by the Centre for Environmental Research and Consultancy (CERC) study (Cope 1995) makes this point very clearly, as shown in Table 8.2.

Some measures have been written into the legislation in an attempt to minimise the possibility of pollution, such as the requirement that all sites, except those taking inert waste, employ a leachate collection system and meet universal minimum liner specifications. However, it is obvious that a method of dealing with waste which removes the bulk of the problem at the outset must be a preferable solution. The use of biological treatment technologies to process wastes has, then, considerable future potential both in direct application to waste management itself and in a number of allied pollution control issues which currently beset this particular industry. Coupled with the twin external driving forces of legislation and

Table 8.2 *General range of values for landfill leachate*

Determinand	Value range
pH	5.5–8
BOD	5–80 000
COD	50–100 000
Nitrate	0.1–1 000
Sulphate	1–1 000
VFAs	150–50 000

Concentrations expressed in mg/l, pH in pH units.

economic forces in the commercial arena, this means that waste biotechnologies seem certain to assume greater importance in the coming decades.

Biological Waste Treatment

The aims of biological treatment are relatively straightforward and can be summed up in the following three points:

1. Reducing the potential for adverse effects to the environment or human health.
2. Reclaiming valuable minerals for reuse.
3. Generating a useful final product.

Broadly speaking, this effectively means the decomposition of the biowaste by microbes to produce a stable, bulk-reduced material, during which process the complex organic molecules originally present are converted into simpler chemicals. This makes them available for literal recycling in a wider biological context.

To some extent these three aims can be seen as forming a natural hierarchy, since removing environmental or health risks, and deriving a stable product, forms the bottom rung of the ladder for all biological waste treatment technologies. Clearly, whatever the final use of the material is to be, it must be safe in both human and ecological terms. The recovery of substances, like nitrogen, potassium and phosphorus, which can be beneficially reused, forms the next level up, and is, in any case, closely linked to stabilisation, because these chemicals, if left untreated within the material, would provide the potential for unwanted microbial activity at a later date. The final stage, the generation of a useful end-product, is obviously dependent on the previous two objectives having been met with some degree of efficiency. The possible uses of the final material, and just as importantly, its acceptability to the market, will largely be governed by the certainty and effectiveness of the preceding processes of stabilisation and reclamation. Thus, while the hierarchical view may, in some ways, be both a natural and a convenient one, these issues are not always as clear-cut, particularly in respect of the implications for commercial biowaste treatment, as this approach might lead one to believe.

In practical terms, the application of this leads to two major environmental benefits. Firstly, and most obviously, the volume of biowaste consigned to landfill is decreased. This in turn brings about the reduction of landfill gas emissions to the atmosphere and thus a lessening of the overall greenhouse gas contribution, while also freeing up space for materials for which landfill genuinely is the most appropriate disposal option. Secondly, good biological treatment results in the generation of a soil amendment product, which potentially can help lessen the demand for peat, reduce the use of artificial fertilisers, improve soil fertility and mitigate the effects of erosion.

As has been mentioned previously, stabilisation is central to the whole of biological waste treatment. This is the key factor in producing a final marketable commodity, since only a consistent and quality product, with guaranteed freedom from weeds and pathogens, will encourage sufficient customer confidence to give it the necessary commercial edge. As a good working definition, stabilisation is biodegradation to the point that the material produced can be stored normally, in piles, heaps or bags, even under wet conditions, without problems being encountered. In similar circumstances, an incompletely stabilised material might well begin to smell, begin renewed microbial activity or attract flies. Defined in this way, stability is somewhat difficult to measure objectively and, as a result, direct respirometry of the specific oxygen uptake rate (SOUR) has steadily gained support as a potential means to quantify it directly. Certainly, it offers a very effective window on microbial activity within the matter being processed, but until the method becomes more widespread and uniform in its application, the true practical value of the approach remains to be seen.

The early successes of biowaste treatment have typically been achieved with the plant matter from domestic, commercial and municipal gardens, often called ‘green’ or ‘yard’ wastes. There are many reasons for this. The material is readily biodegradable, and often there is a legal obligation on the householder to dispose of it separately from the general domestic waste. In the UK alone, the production of this type of biowaste is estimated at around 5 million tonnes per annum (DETR 1999b), making this one area in which biological waste treatment can make very swift advances. Nowhere is the point better illustrated than in the USA, where the upsurge in yard waste processing throughout the 1990s, led to a biowaste recovery rate of more than 40%, which made an effective contribution of nearly 25% to overall US recycling figures. In many respects, however, discussions of waste types and their suitability for treatment are irrelevancies. Legislation tends to be focused on excluding putrescible material from landfills and, thus, generally seeks to make no distinction as to point of origin and applies equally to all forms. The reasons for this are obvious, since to do otherwise would make practical enforcement a nightmare of impossibility. In any case, the way in which waste is collected and its resultant condition on arrival at the treatment plant is of considerably greater influence on its ease of processing and the quality of the derived final product.

There are three general ways in which waste is collected: as mixed MSW, as part of a separate collection scheme, or via civic amenity sites and recycling banks. From a purely biowaste standpoint, mixed waste is far from ideal and requires considerable additional effort to produce a biodegradable fraction suitable for any kind of bioprocessing, not least because the risk of cross-contamination is so high. By contrast, suitably designed separate collection schemes can yield a very good biowaste feedstock, as a number of countries around the world have successfully shown. However, not all separate collections are the same, and they may vary greatly as a result of the demands of local waste initiatives and specific targets for recycling. As with all attempts to maximise the rational use of waste, the delivered benefits of any scheme inevitably reflect the overall emphasis of the project itself. Where the major desire is to optimise the recovery of traditional dry recyclables, biowaste may fare poorly. Systems deliberately put in place to divert biodegradable material from landfill or incineration routes, however, generally achieve extremely satisfactory results. In many respects, the same largely holds true for recycling banks and amenity sites. Dependent on local emphasis, the operation can recover very specific, narrow waste types, or larger, more loosely defined, general groups. Where 'garden' waste is kept separate, and not simply consigned to the overloaded skip labelled 'other wastes', the biowaste fraction produced can, again, be of a very high quality and readily acceptable for biological treatment. Indeed, it is generally accepted that this material is the cleanest source available for processing and it constitutes something in the region of three-quarters of the biowaste treated yearly in the UK (DETR 1999b).

For those approaches to collection which do not involve separation of the putrescible fraction at source, obviously some form of sorting will be required before the material can be taken on to any kind of biological processing. It lies beyond the remit of this work to attempt to describe the methods by which this can be achieved, or their relative merits. Suffice it to say that whatever onsite sorting is used must be matched adequately to the demands of the incoming waste stream, the intended treatment biotechnology and the available local resources. However the biowaste-rich fraction is obtained, the major consideration for processing is its physical form, which is of more fundamental significance to biowaste than any other refuse-reclaimed material. For traditional dry recyclables, chipping, crushing or baling are mere matters of convenience; for biotreatment, particle size, purity and consistency are indivisible from the process itself, since they are defined by the requirements of the microbes responsible. In general terms this means that the biowaste is shredded to break it down into small and relatively uniform pieces, the exact requirements being dictated by the particular treatment technology to be used. This not only makes mixing and homogenisation easier to achieve but also, by increasing the surface area to volume ratio, makes the material more available to microbial action.

There are a number of processes currently available in varying degrees of commercial readiness, and others under development, to deal with biowaste. While the underlying aims and basic requirements of all these biotechnologies are essentially the same, there is some variance of detail between individual methods. Two general approaches in particular, composting and anaerobic digestion, are so well established and between them account for such a large a proportion of the biowaste treated worldwide, that the discussion of specific technologies must begin with them.

Composting

For centuries, gardeners and horticulturalists have encouraged biodegradable waste to break down to produce stable, nutrient-rich compost for use in pots or directly for improving the soil. This application of the natural, exothermic process of aerobic decomposition, is familiar and time-honoured. More recently, however, composting has been the recipient of increased attention as a potential means of treating biowaste on a municipal basis. Though the scale of such operations imposes certain restrictions of its own, generally, putrescible matter decomposes more efficiently and completely when oxygen is readily available. This leads to proteins being degraded to nitrogen or ammonia and ultimately mineralised to nitrate, while fats and carbohydrates are broken down to carbon dioxide and water, via organic acids. This is, of course, purely a mass flow overview of the process since a proportion of the material becomes incorporated into microbial cells, as the decomposers themselves multiply and grow. Even under optimised environmental conditions, there are a number of rate-limiting factors in the process, which include extracellular hydrolytic enzyme production, the speed of hydrolysis itself and the efficiency of oxygen transfer. These may, in turn, be influenced by other aspects such as the particle size and nature of the biowaste material to be treated.

In a practical application, this can be a major consideration as the kinds of biowaste to be composted can vary greatly, particularly when derived from municipal solid waste, since seasonal variation, local conditions and climate may produce a highly heterogeneous material. On the other hand, biowastes from food processing or horticulture can be remarkably consistent and homogeneous. Accordingly, the details of breakdown may be very complex, involving a number of intermediary compounds and different organisms utilising various biological pathways. However, in broad terms, the composting process can be split into the following four distinct general phases, which are chiefly defined by their temperature characteristics.

The composting process

- Latent phase (Ambient temperature–c. 22 °C). Composting microbes infiltrate, colonise and acclimatise to the material.

- Growth phase (c. 22 °C–c. 40 °C). Growth and reproduction of microbes, resulting in a high respiration rate and consequent elevation of temperature to a mesophilic range.
- Thermophilic phase (c. 40 °C–c. 60 °C). Compost pile achieves peak temperature and maximum pathogen sterilisation. At the end of this phase the temperature drops to around 40 °C.
- Maturation phase (c. 40 °C–ambient). Slower, secondary mesophilic phase, with the temperature gradually dropping to ambient temperature as the microbial activity within the material decreases. Complex organic chemicals are transformed into humic compounds and residual ammonia undergoes nitrification to nitrite and subsequently nitrate.

For a municipal scheme, as shown schematically in Figure 8.1, time and space at the facility will be at a premium, so the faster the biowaste can be colonised by a suitable microbial culture, the sooner the treatment space will be ready to accept a new load for processing. Hence the principal focus of environmental biotechnology for the optimisation of conditions for enhanced biological breakdown is in reducing the time-lag inherent in the latent phase. One of the major means to achieve this is to ensure that the material to be treated is presented in as suitable a form as possible. This typically involves some form of grinding or shredding to produce an ideal physical particle size, but biochemical considerations are every bit as important in this respect. At the same time as breaking down biowaste into simpler compounds, the process also brings about a change in the carbon to nitrogen (C:N) ratio of the material, as substantial quantities of organic carbon are converted to carbon dioxide. The initial C:N ratio is an important factor in the success of composting, since a ratio much more than 25:1 can inhibit the mineralisation of nitrogen and adversely affect the product's final maturation. This latter aspect has clear implications for any intended use of the end-product as a fertiliser or soil enhancer, particularly for a large-scale, commercial operation. To take account of this, facilities accepting mixed-source waste for composting, often find it necessary to undertake a measure of mixing and blending to ensure

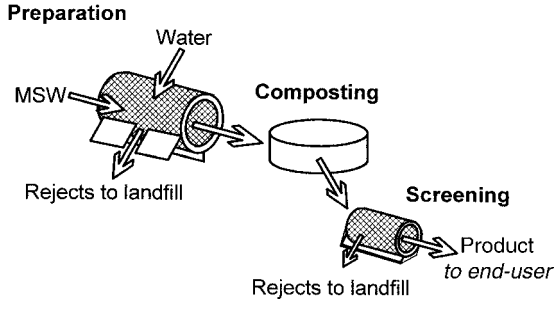

Figure 8.1 *Compost plant schematic flow chart*

Table 8.3 *Illustrative carbon to nitrogen ratios*

Material	C:N
Food wastes	15:1
Sewage sludge (digested)	16:1
Grass clippings	19:1
Cow manure	20:1
Horse manure	25:1
Leaves and foliage	60:1
Bark	120:1
Paper	170:1
Wood and sawdust	500:1

an appropriate balance. It is possible to categorise different kinds of biowaste according to their carbon/nitrogen content, as illustrated in Table 8.3.

Most plants obtain the nitrogen they require as nitrate. The mineralisation of nitrogen is performed by two genera of bacteria, *Nitrosomonas*, responsible for converting ammonia to nitrite and *Nitrobacter* which completes the nitrification. Inactivated at temperatures above 40 °C and with a relatively slow growth rate, their activity is largely confined to the maturation phase. Thus, the proper mineralisation of nitrogen can only be achieved after the growth and thermophilic stages have been completed, which themselves require a suitable C:N balance in the initial feedstock in the first place.

A number of different organisms are involved in composting, including bacteria, fungi, protozoa, mites, nematodes, insects and annelids, there being a natural succession of forms allied with the four phases of the process. Thus the initial decomposition is brought about by mesophilic bacteria in the main, until their increased activity raises the temperature into the range favoured by thermophilic organisms. These thermophiles then play a major role in the breakdown of carbohydrates and proteins, before they themselves become inhibited by the 70–75 °C heat of the composting pile. Later, as the temperature begins to drop, actinomycetes become the dominant group, giving the ageing compost a characteristic white-grey appearance. Although largely confined to the surface layers, they are of considerable importance in the decomposition of cellulose and lignin, which are two of the more difficult components of biowaste to break down.

The microbial component of compost is an area of particular future potential, particularly as a measure of product quality. While simple chemical analysis has traditionally been used to assess composts, concentrating on NPK values and placing it on the same footing as artificial fertiliser, there has been a growing realisation that its complex nature means that this does not tell the full story. The potential benefits in terms of soil flora improvement and plant pathogen suppression cannot be inferred from a compost's gross mineral contribution and so, in an attempt to produce a more comprehensive yardstick, some producers and

users have begun to investigate assessment based on microbiological profiling. Pioneering work in the USA, by BBC Laboratories of Arizona, has led to the development of the first predictive tool for the value of a compost as a soil microbial inoculant, based on the concentrations of six key classes of micro-organisms present (Bess 1999). The marketing of biowaste-derived products is made more difficult by a number of factors which lie outside of the scope of this work, most particularly a lack of a recognised and universally agreed standard. The application of microbiological criteria, in conjunction with the likes of mineral analysis and maturity assessment, could lead to significantly better overall characterisation of composts. Moreover, there seems no reason why this approach should not be extended to all biowaste-derived soil amendments, enabling direct comparisons to be made and the suitability of any given product for a particular use to be measured objectively.

Applying Composting to Waste Management

Composting has an appeal to local authorities needing to meet diversion targets while keeping a watch on their budgets, since it is relatively simple and does not demand particularly high resource investment, either to set up or run. As a consequence, many of the initiatives instigated to deal with biowaste have been based on composting of one form or another. In the broadest of terms, such schemes fall into one of two categories, namely, home composting, or centralised facilities. The focus of this section will fall on the latter, as a more representative application of biotechnology, though to set this in context, it is worth giving a brief outline of the former.

Home composting

Home-based systems differ little in reality from the traditional gardener's approach, putting biodegradable material into a heap or, more typically a bin, often provided free or at a subsidised price, by the local council. Though this does have the advantage of directly involving people in the disposal of their own waste and the informality of this approach has its own advantages, such schemes are not without certain drawbacks. To work, these initiatives draw heavily on householder goodwill and competence, not to mention a good choice of bin and simply making the means available does not, of course, ensure that it will be used. Anecdotal evidence suggests that many bins lie unused within two years, once the initial enthusiasm wears off, and an investigation into Luton's trial scheme suggests that home composting may make little difference to the overall amount of waste generated (Wright 1998). The kind of instant minimisation popularly supposed would seem to be far from guaranteed.

One clear advantage that household composters do have, however, is the ability to control very closely what goes into their system. This avoids both the issues

of contamination and the need for post-user segregation typically foisted on the operators of centralised facilities. Thus, although domestic initiatives of this kind are unlikely ever to make the sort of difference to biowaste treatment demanded by legislation on their own, it seems likely that they will always have a role to play, perhaps most especially in remoter areas where collection for processing elsewhere might prove uneconomic.

Centralised composting

The biochemistry and microbiology of all composting remains essentially the same, irrespective of the details of the operation. However, the scale of schemes set up to deal with a municipal biowaste stream in terms of the physical volume involved imposes certain additional considerations, not least amongst them being the need to ensure adequate aeration. In the back-garden compost heap, oxygen diffuses directly into the material; large-scale composting cannot rely on this method, as the large quantities involved lead to a lower surface area to volume ratio, limiting natural oxygen ingress. To overcome this, various techniques make use of mixing, turning or pumping, but, clearly, the additional energy required has its own implications for a commercial operation.

Approaches suitable for municipal scale use fall into five main categories:

- windrow;
- static pile;
- tunnel;
- rotary drum;
- in-vessel.

A sixth form, termed 'tower composting', may occasionally be encountered, but it is generally much less common than the other five.

No one system is the universal ideal. The decision as to which approach is likely to be the most suitable for specific conditions is dependent on a number of factors, including the nature and quantity of biowaste available, the required quality of the end-product, the time available for processing, local workforce and land availability and financial considerations. In the brief descriptions of each system which follow, the main features are set out, but it is important to realise that there are many other issues which have a bearing on the selection of appropriate technologies than the necessary brevity of this chapter permits us to explore fully.

Windrow

The biowaste is laid in parallel long rows, around two or three metres high and three or four metres across at the base, forming a characteristically trapezoid shape. Windrowing is usually done on a large scale and, though they can be situated under cover, generally they tend to be outdoor facilities, which exposes them

more to the vagaries of the weather and makes process control more difficult. While this might be a problem for some kinds of biowaste, for the typical park and garden waste treated by this method, it generally is not. However, some early attempts were prone to heavy leachate production in conditions of high rainfall, leading to concerns regarding localised soil pollution. This was largely an engineering problem, however, and the almost universal requirement for a suitably constructed concrete pad and interceptor has made this virtually unknown today.

Limited aeration occurs naturally via diffusion and convection currents, but this is heavily augmented by a regime of regular turning, which also helps to mix the composting material, thus helping to make the rate of breakdown more uniform. Dependent on the size of the operation, this may be done by anything from front-end loaders on very small sites, to self-propelled specialised turners which straddle the windrows at larger facilities. The intervals between turning can be tailored to the stage of the process, being more frequent early on, when oxygen demand is high, becoming longer as composting proceeds.

Windrows have a typically high land requirement, can potentially give rise to odour problems and are potentially likely to release fungal spores and other bioaerosols during turning. Despite these drawbacks, this approach accounts for the vast majority of centralised composting projects, possibly because it is often carried out as an addition to existing landfill operations, thereby significantly reducing the actual nuisance generated.

Static pile

Superficially resembling the previous method, the static pile, as its name suggests, is not turned and thus does not have to conform to the dimensions of a turner, allowing the rows to be considerably taller and wider. What mixing is needed can be achieved using standard agricultural equipment and so these systems tend to be significantly cheaper in respect of equipment, manpower and running costs. They do not, however, remove the land requirement, since decomposition progresses at a slower rate, causing the material to remain on site for a longer period.

In an attempt to get around this, a variant on the idea has been developed, particularly for the co-composting of food or garden biowaste with manure or sewage sludge, which relies on forced aeration. With a perforated floor and fans to push air through the material, the characteristically low oxygen level within the core of traditional static piles is avoided and processing accelerated. However, bulk air movement is expensive, so this system tends to be reserved for small tonnage facilities, often in areas where good odour control is of major importance.

Tunnel composting

Tunnel composting has been used by the mushroom industry for a number of years, where processing takes place inside closed tunnels, around five metres high and up to 40 feet in length. There has been some interest in adapting it to

deal with MSW-derived material and one system which has evolved uses huge polythene bags, a metre or so high and 60-metres long into which a special filling machine packs around 75 tonnes of source separated putrescible material. This particular design also makes use of a fan to force air through the material rather like the previous technique, with slits in the side wall allowing carbon dioxide to escape.

The processing time is reduced compared with a similar sized aerated static pile, since the environmental conditions within the tunnel are easier to control, though similar cost considerations apply.

Rotary drum

Rotary drums seem to drift in and out of fashion, often being favoured by those needing to co-compost sewage sludge with more fibrous material, like crop residues, straw or garden waste. The principle is simple; the waste is loaded into the drum which then slowly rotates. This gently tumbles the material, mixing it and helping to aerate it. The drums themselves are usually steel, insulated to reduce heat loss.

In-vessel

Sometimes also called closed reactor composters, there are a number of designs of in-vessel systems available, ranging from small steel or plastic tanks, through larger metal cages to long concrete troughs with high sidewalls. The main characteristic of these systems is that the waste breaks down within an enclosed container, which allows the internal environmental conditions to be closely controlled. This approach offers a very efficient use of space and close regulation of the process, since some form of mechanical aeration is also required it is significantly more expensive on a tonne for tonne basis than the less resource-intensive methods. Accordingly, it is less suitable for large capacity requirements, it has a role in smaller scale operations or where the material to be treated does not easily fit into other kinds of processing or disposal arrangements.

This is less of a natural group than the preceding approaches to composting, since it encompasses far greater variety of design. Consequently there is a marked variance in the capacity, complexity and cost of these systems.

Process parameters

Aside from aeration, which has already been discussed, a number of other parameters affect the composting process. Although these are themselves influenced to some extent by the method being used, in general the most important of these factors are:

- temperature;
- moisture content;

- particle size;
- nature of the feedstock;
- accelerants;
- processing time.

Temperature

The temperature changes over the stages of composting have important implications for the efficiency of the process. It is widely agreed that for satisfactory sanitisation, the material should reach at least 55 °C, though there is less of a consensus over the required duration of this exposure.

On the other hand, the temperature should not be permitted to exceed 70 °C, since above this most of the compost microbes either die off or become inactivated, causing the biological breakdown to slow or stop. In a commercial operation, lost processing time has inevitable financial consequences.

Moisture content

A moisture content of around 60% is the ideal target for optimum composting, though anything within a range of between 40–70% will suffice. While some biowastes meet this requirement naturally; others forms can be surprisingly dry, sometimes with a moisture content as low as 25–30%, which approaches the levels at which severe biological inhibition can occur. Equally, too wet a material may be a problem as this may restrict aeration and even encourage leaching. Even when the initial mix is right, composting matter gradually loses moisture over time and evaporative losses from the surface of the composting biowaste can cause problems, especially in frequently turned windrow regimes. Careful monitoring and appropriate management is necessary to ensure that the optimum range is maintained.

Particle size

The optimum particle size for composting is, of necessity, something of a compromise. The smaller the individual pieces, the larger the surface area to volume ratio, which makes more of the material available to microbial attack, thus speeding up the process of decomposition. However, particles which are shredded too finely will tend to become compacted and so reduce aeration within the material. Consequently, a balance must be struck, providing the smallest possible particle size which does not interfere with air flow. Individual design features may need to be considered; dependent on the system used, bed depth, aeration method and the nature of the biowaste itself can all have an influence.

Nature of the feedstock

The importance of the carbon to nitrogen (C:N) ratio and the need for careful management to ensure a proper balance has already been discussed. In addition,

for some materials, the use of amendments or co-composting with other wastes can also help optimise conditions for biological treatment. Sewage sludge and manures are often used in this way, but they can also boost the available nutrient levels, often in an uncertain way and by variable amounts. Generally, additives are used where there is a need to improve either the chemical or the physical nature of the composting material. Clearly, for a large commercial operation, it is essential that whatever is used does not significantly affect the economics of the plant and for this reason, although artificial fertilisers are an ideal way of increasing nutrient content, they are seldom used in household waste applications. Their expense relative to these low-cost biowastes effectively rules them out; they are, however, often to be seen in *ex situ* bioremediation operations, since composting contaminated soil commands a higher price.

Additions to the original material typically accelerate processing, but careful monitoring is essential since the blend may exhibit very different decompositional characteristics, which may ultimately influence the nature of the final product derived.

Accelerants

Although gardeners have a number of proprietary brands of compost accelerants available to them, this is not an approach often used at commercial facilities, mainly due to the scale of these operations and the consequent expense. As with nutrient addition, this tends to be reserved for use on high value wastes, though many common substances used in co-composting programmes, like manures, are themselves widely accepted to act as natural accelerants. Though their effect is more variable, it seems likely that this is the only form of enhanced processing applicable for general biowaste use.

Processing time

In many respects, the time required is a function of all the other factors. Processing garden or food waste can be achieved in under three months using aerated, in-vessel or turned windrow systems, while in a simple static pile, it may take a year or more to reach the same state. Inevitably much depends also on the management regime, since process optimisation is the key to accelerated biotreatment and good operation practice is, consequently, of considerable importance.

Anaerobic Digestion

Although composting certainly accounts for the majority of biowaste treatment applications around the world, anaerobic digestion (AD) is an alternative option which has been receiving increasing interest over recent years. In many respects, it is a regulated version of the natural events of landfill, in that it results in the

controlled release of methane-rich biogas, which offers the potential for a very real form of energy from waste. This technology is viewed in certain circles as rather novel, but this is not really the case. It has been used in the water industry for around a hundred years to treat sewage and, more recently, been successfully applied to the processing of agricultural and household wastes, most notably in Germany and the Netherlands. However, waste management tends to be a naturally cautious field and the relative lack of a proven track record with MSW-derived biowaste compared to composting has made the uptake of this approach slow.

The key to effective practical applications of AD technology lies in regulating and optimising the internal environment of an enclosed bioreactor vessel such that the ideal conditions for the process are produced and maintained. Under these circumstances, in the absence of free oxygen, anaerobic bacteria convert the large organic molecules mainly into methane CH_4 and carbon dioxide CO_2.

The actual progression of this breakdown is chemically very complex, potentially involving hundreds of intermediary reactions and compounds, many of which have their own additional requirements in terms of catalysts, enzymes or synergistic chemicals. Unlike composting, AD occurs at one of three distinct temperature ranges, namely:

- Cryophilic ($<20\,^{\circ}C$).
- Mesophilic ($20–45\,^{\circ}C$).
- Thermophilic ($>45\,^{\circ}C$).

Since AD is very much less exothermic than composting, within a landfill or in bogs and swamps, it proceeds under cryophilic conditions. This largely accounts for the relatively protracted timescale and the irregular progress of breakdown typically encountered in these examples. In order to overcome these drawbacks, engineered anaerobic bioreactors are usually run at one or other of the higher ranges, with additional heat supplied by external means to elevate the temperature to the required level. A variety of technology vendors have developed commercial systems based around either thermophilic or mesophilic digestion, which have their own particular characteristics. Without entering into a lengthy discussion of the relative merits of these approaches, it is important to note that the internal conditions favour different bacterial complements and that certain aspects of the reaction details also differ. Consequently, for any given application, one or other may be particularly suited, dependent on the specifics of the material to be processed and the overall requirements for treatment.

The digestion process

Hydrolysis

Carbohydrates, cellulose, proteins and fats are broken down and liquefied by the extracellular enzymes produced by hydrolytic bacteria. The proteins are broken

down into amino acids, fats into long-chain fatty acids and carbohydrates into simple sugars, while the liquefaction of complex biological polymers, especially cellulose, to simple, soluble substances is often the rate-limiting step in digestion. The rate of hydrolysis is governed by the nature and availability of the substrate, bacterial population, temperature and pH.

Acidogenesis

The monomers released by hydrolysis, together with volatile fatty acids (VFAs) derived from the protein, fat and carbohydrate components of the material being treated are converted to acetic, lactic and propionic acids, the pH falling as the concentrations of these rise. Methanol and other simple alcohols, carbon dioxide and hydrogen are also produced during acidogenesis, the exact proportions of the different byproducts being dependent on bacterial species and the environmental conditions within the reactor. Though we have considered these events as a single stage in the process, some authorities prefer to subdivide them into *acidogenesis* and *acetogenesis* to highlight the importance of acetic acid, which accounts for around 75% of the methane produced by the next step.

Methanogenesis

Relying on obligate anaerobes whose overall growth rate is slower than those responsible for the preceding stages, this final phase involves the production of methane from the raw materials previously generated. Of these, acetic acid and the closely related acetate are the most important, for the reason mentioned above. There are other potential substrates for methanogenic bacteria, but we will limit the scope of this discussion to the most usual examples, as represented in the following equations:

Acetic acid

$$CH_3COOH \longrightarrow CH_4 + CO_2$$

Methanol

$$CH_3OH + H_2 \longrightarrow CH_4 + H_2O$$

Carbon dioxide and hydrogen

$$CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O$$

Methanogenic bacteria also play an important part in the wider overall breakdown process, since by converting volatile fatty acids (VFAs) into methane, they effectively act to limit pH decrease within the digester. With the acid/base equilibrium naturally regulated in this way, any potential bacterial inhibition by acidification is effectively overcome. This is particularly important for methanogens themselves,

since they thrive in a relatively narrow pH threshold of 6.6–7.0, becoming progressively more impaired as the pH falls below 6.4. In this event, the persistence of unmodified VFAs can have potentially serious implications for the final use or disposal of the material derived.

There are four main groups of bacteria involved in AD, as shown below, with some typical examples of each:

- Hydrolytic fermentative bacteria – *Clostridium* and *Peptococcus*.
- Acetogenic bacteria – *Syntrophobacter* and *Syntrophomonas*.
- Acetoclastic methanogens – *Methanosarcina* and *Methanothrix*.
- Hydrogenotrophic methanogens – *Methanobacterium* and *Methanobrevibacterium*.

In reality, these are not the only species present in a digester and, though the stages previously described represent the main desired biochemical reactions, a number of additional bacterial types and biochemical pathways play a role in the overall breakdown process. As with composting, there is much interaction between these various organisms.

Applying AD to Waste Management

The nature of anaerobic digestion inevitably means that its applications to waste management are relatively large-scale operations, there being no effective equivalent of home composting. Hence, whether the application is as an onsite treatment for process effluent or as part of a centralised municipal waste initiative, the approach relies heavily on engineering, a schematic plant being shown in Figure 8.2. This is in clear contrast with composting and, together with the attendant additional costs, probably goes further to explain the overall lower

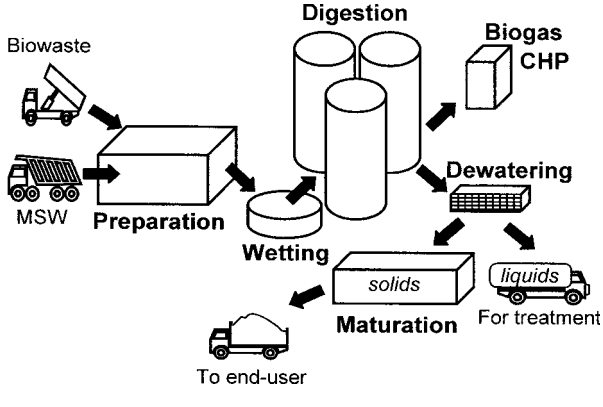

Figure 8.2 *AD plant schematic flow chart*

take-up of this technology than any other factor. It should also be apparent that more resources, and primarily a more skilled workforce, are essential prerequisites for success. However, for wastes which are particularly suited to this form of biotechnology, a number of cost-benefit analyses over the years have shown that these drawbacks may often be outweighed by the advantages inherent in the system. As with so many practical applications of environmental biotechnology, there is seldom one catch-all solution and the most appropriate approach can only really be judged on the specifics of the problem. There will always be cases when either composting or AD is self-evidently the most suitable route; when the matter is less clear-cut, however, the technology decision is often much more difficult to make.

There are many ways in which anaerobic digestion systems may be categorised as will be briefly discussed below. However, it is important to realise that, irrespective of their individual construction, they all fundamentally consist of isolated vessels of some kind, designed to exclude air and maintain internal conditions at the optimum for bacterial action. It is possible to describe systems treating slurries of 15% total dry solids (TDS) or less as 'wet', or 'dry' if their TDS exceeds this figure. Alternatively, the temperature range at which they are operated can be used, thus leading to defining AD systems as either mesophilic or thermophilic.

The loading regime adopted can also be a useful means of distinguishing digester types for some purposes, allowing a distinction to be drawn between 'batch' and 'continuous' systems. The former are filled in a single go, then permitted to digest the contents before being emptied and recharged, while the latter have a continuous cycle of new biowaste being added and processed material being drawn off. Figure 8.3 shows the interrelationship of these various branches of the AD family tree.

However these are, in effect, operational criteria and as such, though useful in themselves, they can tend to unite dissimilar technologies within essentially

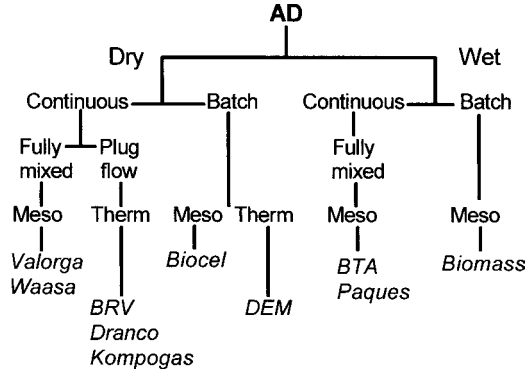

Figure 8.3 *Classifying anaerobic digestion systems*

artificial groupings, giving little clue as to which best suits what type of biowaste. For this, an examination of aspects of the digester design and engineering principles can often provide a better insight, as in the following descriptions of some of the major examples.

Anaerobic baffled reactor (ABR)

These generally feature a horizontal flow of biowaste through the digester vessel, and are suitable for a wide range of materials. The Valorga process, with its patented gas recirculation and mixing system, is based on this approach.

Anaerobic fixed film reactor (AFFR)

These digesters have a fixed growth plate on which a bacterial biofilm is established, digestion taking place on this surface. They are ideal for relatively weak biowastes with low solids content, but are of less use in other applications.

Completely mixed contact reactor (CMCR)

In this design, the biomass derived during processing is recycled after dewatering to increase the retention time for the solids. This approach is typically used to treat high strength, industrial biowastes.

Continuously stirred tank reactor (CSTR)

Intended to treat slurries and liquid biowastes, this system is essentially the same as the preceding CMCR design, but without the need for the solids' recycle.

Fluidised bed reactor (FBR)

Sometimes termed the expanded bed reactor, this system relies on an internal microbial growth medium, which is fluidised by the waste liquid circulating within it. Accordingly they are only suitable for liquid biowastes or very dilute slurries.

Multi-phasic processes (MPP)

Physically separating the stages of AD into different reactors, these systems are principally of use as experimental tools for increasing understanding of the pathways and mechanisms of the anaerobic digestion process. They are featured here for completeness, but they do not generally have any commercial application.

Upflow anaerobic sludge blanket (UASB)

These systems hold relatively high numbers of active bacteria, which makes them suitable for treating biowastes of low solids content and they are commonly used to process high-strength industrial waste liquids or light suspensions.

From all of this discussion, it should be obvious that, however proprietary AD technologies are classified, no one type is universally ideal or superior, each having certain characteristics which make it appropriate for particular wastes and less suitable for others. This means, of course, that although comparisons of the various approaches are of great interest to potential users, in practice they are difficult to make in any meaningful way. Even certified data from an operating plant can only be taken as broadly indicative of how a similar one might perform elsewhere, especially in respect of breakdown efficiency and biogas generation or quality.

Process parameters

Efficient AD requires the development and maintenance of an optimised internal environment to facilitate biological activity. This is of particular importance in the commercial setting and a number of both physical and chemical factors must be taken into account to achieve it, of which the most important are:

- temperature;
- retention period;
- agitation;
- wetness;
- feedstock;
- loading rate;
- pH and volatile fatty acids concentration.

Temperature

As mentioned previously, in commercial systems, digesters are operated at around 35 °C (mesophilic) or 55 °C (thermophilic). Irrespective of which approach is adopted for any particular application, a relatively constant temperature is essential for the process to run at its greatest efficiency.

Retention period

Although the amount of biowaste degraded depends on its character, the availability of bacteria and the time allowed for processing, temperature governs both the rate of breakdown itself and the particular bacterial species present in the digester. Hence, there is a direct relationship between temperature and the retention period. Some AD technologies have attempted to shorten the retention period by separating the stages of the process within the digester. The separation of the acidogenic and methanogenic stages permits each to be optimised and this has been well demonstrated at laboratory scale using a completely mixed digester, with phase-isolation being achieved by pH manipulation. Despite the greater efficiency, higher biogas yield and enhanced process stability claimed, it has seen

little large-scale use, probably as a result of the higher cost implications of such a system.

Agitation

The agitation of the digester contents has a number of benefits, one of the most obvious being that it helps to mix up material, evening out any localised concentrations, thus also helping to stop the formation of 'dead zones' or scum. In addition, it increases the waste's availability to the bacteria, helps remove and disperse metabolic products and also acts to ensure a more uniform temperature within the digester. There have been some suggestions that efficient mixing enhances methane production, but the evidence is inconclusive, so it seems likely that this may only be of noticeable benefit for some systems or operational regimes.

Wetness

Anaerobic digestion is a wet process and any biowaste which is too dry in its natural state will require the addition of a suitable liquid, typically water, recycled AD process liquor or slurries, either sewage or agricultural, before processing can begin. In order to minimise digester size, so-called 'dry' systems have tended to dominate the commercial world, but the relatively thicker contents inevitably demand more energy to mix effectively, off-setting much of the advantage. Comparisons of 'wet' or 'dry' approaches, like those of mesophilic or thermophilic processes, generally yield no clear winner. Each system has particular advantages and applications for certain kinds of biowaste, and selecting the right one for any given use is almost always best done on this basis.

Feedstock

As with composting, the particle size and nature of the material to be treated play an important role. The ease of breakdown is largely defined by the characteristics of the biowaste material to be treated, but generally finer particles allow for better processing and a homogeneous slurry or suspension is the ideal feedstock for AD. It must be stressed, however, that some biowaste types, particularly the likes of lignin-rich, woody material, are relatively resistant to this process.

Loading rate

Loading depends on the characteristics of the waste, its degree of wetness, digester volume, the expected retention period and similar system design parameters. It is typically expressed as the chemical oxygen demand per cubic metre of digester void-space (COD/m^3) or, for continuous or semi-continuous process, per unit time (COD/day, COD/hr).

pH and volatile fatty acids concentration

These are interdependent factors which need to be considered together. Adequate process control and digester optimisation requires suitable pH monitoring, since many of the bacteria involved are pH sensitive. In particular, acidogens, having better tolerance to acidity, may produce acids faster than the increasingly inhibited methanogens can use it, in conditions of low pH, leading to spiralling acidity and the potential for process collapse. A number of acid–base reactions exist within the typical AD process, which lead to a measure of natural, inbuilt resistance to major pH swings. However, under certain circumstances, the need for external interference may arise and the amount of such intervention necessary to maintain proper equilibrium will depend on the nature of the material. For some wastes, pH control may only be necessary during start-up or in overload conditions; for others where acidity is habitually shown to be a problem, continuous control may be necessary.

Volatile fatty acid concentration is one of the most important process indicators. Elevated VFAs are characteristic of AD instability and thus they may be the first indication of a developing problem, though the actual cause may be less immediately obvious. Inadequate mixing, excessive loading, poor temperature control or bacterial inhibition can all lead to an increase in VFAs and a decrease in pH. Considering the inconvenience and cost of being forced to empty a sick reactor, commercial AD operations rely greatly on routine monitoring of this kind.

Biogas

The production of methane-rich biogas, which is an essential characteristic of anaerobic digestion, has been used as an important marketing tool, since the generation of energy from waste by biological means has much attraction, not least amongst those countries with a popular dislike of incineration. A cohesive discussion of bioenergy appears in a later chapter and accordingly, a consideration of the wider implications of biogas fuel will be deferred until then. However, it is worth pointing out in the waste-specific context that, though much has been made of the apparent dual benefit of biowaste treatment *and* energy production, in an actual application, it is a practical impossibility to optimise both simultaneously.

Other Biotechnologies

Although composting and anaerobic digestion between them account for by far the bulk of biological waste treatment globally and each has a well-established track record, as is so often the case with practical applications of environmental biotechnology, neither is a clear winner. Dependent on the specifics of the situation, the particular waste composition, local factors and so on, either can have clear benefits to offer and, as we have seen, both can form adequate responses

to the growing demand for biowaste diversion. At the same time, each has its limitations also. For AD, the airtight nature of the reactor, the gas-handling arrangements needed to guarantee that the potentially explosive methane produced can be safely managed and the demands of internal environmental control contribute heavily to the overall capital cost of the plant. In addition, on a practical note, there are certain inherent limits on the levels of contamination by other waste fractions that can be tolerated. Clearly, for some applications, these factors may prove major barriers to use.

On the other hand, composting is essentially less of an engineered solution and in many of the versions often seen at local authority sites, it is a very simple process. The major practical limitation, at least as a sole method of bulk treatment, lies in the physical amount of material, since the typical retention period for composting is longer than AD and the final volume of product derived is greater. Consequently, a relatively large area of land is required for processing and a sizeable market capacity or disposal arrangement is necessary for the compost. In certain circumstances, these issues may be significant blocks to its adoption.

Applications for which neither of the major technologies is an immediately easy fit have, at times, led to interest in the potential of other methods of biowaste treatment. It is beyond the scope of this book to discuss the wider political and economic issues surrounding biowaste initiatives, though it should be clear that such factors commonly play an indivisible part in their implementation. Suffice it to say that these local modalities can often form the most critical deciding factors in determining the suitability of a given approach. This is something of a mixed blessing, since, though it can make direct comparisons between individual methods exceedingly difficult to do in a meaningful way, it does leave space for novel or less well-known technologies to play a role.

Annelidic conversion (AC)

The use of a variety of annelid worm species is one alternative approach that has received fairly regular reawakenings of interest over the years, having been variously termed *worm composting*, *vermicomposting, vermiculture* or our preferred *annelidic conversion*, a term first attributed to H. Carl Klauck of Newgate, Ontario. The description *worm composting* and its like is somewhat misleading, since the process from both biological and operational criteria is quite distinct from true compost production in two significant ways.

Firstly, as we have seen, in traditional composting, breakdown is brought about by the direct actions of a thriving microbial community. Within a worm-based system, while micro-organisms may contribute in some way to the overall biodegradation, their role in this respect is very much incidental to that of the worms themselves.

Secondly, in worm systems, biowaste is typically laid in much shallower layers than is the case for windrows or static piles, frequently being deposited on the

surface of an underlying soil bed. This is a major difference, principally because it reduces the natural self-heating tendency within the decomposing matrix.

Worms of various species can be present in traditional compost heaps, even in thermophilic piles, but they avoid the genuinely thermophilic core, being found at the significantly cooler edges of the heap. In addition, under such conditions the resident annelid population is, in any case, many magnitudes smaller than in the deliberately high-biomass levels of AC systems. While in common with all poikilothermic organisms, worms do require some warmth to remain active, which for most species means a lower limit of 10 °C, they do not generally tolerate temperatures in excess of 30 °C and death occurs above 35 °C. Most species have an optimum range of 18–25 °C, which makes the point very clearly that the highly exothermic conditions encountered as part of the 'true' composting process would be impossible for them to survive, and certainly not in any sizeable numbers.

Annelidic conversion is similar to composting in the sense that it can be scaled to meet particular needs and, as a result, it has been promoted in various forms for both domestic and municipal applications over the years. Again, like composting, particularly in respect of home bins, this has not been entirely free of problems, since all the difficulties regarding bin design, operator diligence and issues of compliance apply if anything, more rigorously to AC as to traditional composting. While some recycling officers have found that these projects have been widely welcomed and effective, others report 'considerable' drop-off rates in usage.

In the case of commercial scale treatment, worm systems have sufficient inbuilt flexibility to be tailored to suit. However, since the beds must be significantly shallower than an equivalent windrow, accommodating the same amount of material for treatment necessitates a much larger land requirement, which may itself prove a constraining factor. Thus, for each tonne of biowaste to be deposited weekly, the typical bed area required is around 45 m^2. Hence, for a typical civic amenity site annual production of 4000 tonnes, and allowing for the seasonal nature of its arising; around half a hectare, or one and a quarter acres, of ground is required simply for the beds themselves. This rises to more than double to provide the necessary service access between and around the wormeries.

Worm systems are essentially biomass intensive, with an initial population density, typically exceeding 500 animals per square metre and a cumulative annelid biomass production rate, once established, of 0.07 kg/m^2. Clearly, this demands careful control of the local environmental conditions within the beds for optimisation of system performance, particularly since the physical and biological needs of the organisms involved lie within more precisely defined limits than those of the microbes responsible for composting. Bed design is partly influenced by the temperature tolerances discussed previously, but the large surface area to volume ratio typical of this method also allows for the ready aeration of the biowaste matrix, especially in the surface layers, where many of the worm species used preferentially reside. Design is further constrained by the need for adequate moisture to permit gas exchange across the annelid skin, which must be

achieved without waterlogging, since this reduces pile aeration and may, further actively drive the resident worms to leave in search of drier conditions. Unsurprisingly, many commercial scale systems make use of extensive drainage works to help avoid this. Well-ventilated covers are also often used, particularly in outdoor installations, which help overcome the rigours of the weather while also producing continuously dark conditions. Accordingly, the worms are encouraged to be active for much more of the day than would otherwise be the case. This brings additional bonuses, since the burrowing of the animals themselves both promotes enhanced aeration and has beneficial effects on odour control, particularly in respect of sulphide concentrations, which have been reported as being reduced by a factor of 100 or more.

In common with trends in many other biotechnological interventions, there has been some interest over the years in developing in-vessel systems. This principally arises as an attempt to create circumstances in which process control can be maximised, but has the additional benefit of also giving rise to a modular and highly portable approach, which has helped annelidic conversion penetrate areas that might otherwise have remained closed to it. One of the reasons for this is that this approach gets around the need for a permanent installation, which may be an important consideration for some applications. However, the unit processing cost is consequently higher than would be the case for a simple land-based system of similar operational capacity.

A number of different species of worms are used in vermicultural operations around the world, but in general terms all of them can be placed into one of two broad categories, namely redworms and earthworms. Although some uncertainty exists as to the absolute validity of this division, it is a useful tool, at least at the functional or morphological level, to aid understanding of the whole approach. The true earthworms are burrowers, and generally speaking consume dead biological material from within the soil itself rather than directly assimilating the biowaste. The nutrient value of the organics so treated is returned via worm casts. Hence, initiatives reliant on earthworms are probably best regarded as a form of worm-enhanced composting. By contrast, redworms, which are also sometimes termed *manure* or *compost* worms, rapidly and directly feed on the biowaste, consuming half or more of their own body weight per day. This influx of material is turned into increased worm biomass, both in terms of individual growth and population increase. As a result, annelidic conversion has tended to make use of redworm species such as *Dendrobaena, Helodrilus* and especially *Eisenia* as the mainstay of these operations. In nature, these animals are naturally found amongst the fallen plant material of woods and forests, where they are commonly associated with the production of leaf litter. Their use in worm beds stands as another example of making use of an organism's natural abilities to achieve the biotechnologist's desired result. When the artificial environment of the worm bed is well enough managed and conditions suitably optimised, the redworms decompose and mineralise the biowaste

extremely efficiently, effectively reprising their role in nature under these engineered conditions.

Despite the predominance of redworms within vermiculture for the reasons outlined, there have been a number of cases that have used true earthworm species, with varying degrees of success. Thus, members of the genera *Lumbricus* and *Amynthas* have featured, but perhaps one of the most notable successes was with *Pheretima elongata*, a deep burrowing worm, native to India, which was used to great effect as part of the Bombay plague prevention project, which was founded in 1994 in response to an earlier outbreak of the disease. The growing amount of waste in the city had been strongly implicated in attracting and harbouring the vector rats. The use of vermiculture proved very effective in reducing the biowaste problem (Menon 1994) and hence was seen as a major preventative measure against a recurrence of the plague.

The use of worms to break down waste is not a particularly novel idea, though like many aspects of environmental biotechnology, it frequently seems to fall first in and then out of favour on a fairly regular basis. The advantages of worm-based biowaste treatment are fairly easy to see. In the first instance, vermiculture offers a high potential volumetric reduction, often exceeding 70% while producing a well-stabilised final product. Secondly, this product itself is rich in potassium, nitrogen, phosphorous and other minerals, which is presented in an ideal form for plant uptake, and hence represents a high fertiliser value. The market for this product has already been successfully established in some parts of the world and many others seem set to follow. This is an important issue, as it has been estimated that for every one tonne of biowaste deposited on the bed, around half a tonne of worm casts is produced (Denham 1996).

Finally, the rapid growth potential of worms under idealised conditions has the potential to provide a harvestable biomass resource, typically either as seed populations for other vermiculture operations or for direct sale into the fishing market. The reputation of the bait outlet has been tarnished in the light of various spectacular collapses, most notably the Californian pyramid franchise back in the 1970s and other similar operations in the UK more recently. Often aimed at farmers seeking diversification ventures, it is unlikely that worm production will truly be the salvation for all of them. However, the fact remains that there are a number of long-established businesses in Britain, the USA and elsewhere successfully trading in live worms for various purposes.

The sequential combination of various approaches into treatment trains has become one of the major themes of environmental biotechnology over recent years. Annelidic conversion has particular potential for use in this way and this may prove of increasing importance within the waste management industry in the future. There is a particularly logical fit between this method and traditional composting, since a period of precomposting permits the thermophilic inactivation of pathogens, while secondary worm action offers a high quality product more rapidly.

There are many of advantages to this method, most obviously in that it allows for the input biowaste to undergo established sanitisation procedures without detriment to the worms themselves, which are, as discussed previously, temperature sensitive. A less commonly appreciated benefit of this approach is that the period of initial composting significantly reduces worm ammonia exposure, to which, again, they are very sensitive. However, as with so much of these combined approaches, there is a need to manage the treatment conditions carefully to produce the optimisation desired. There is evidence to suggest that a precomposting phase has a negative effect on worm growth and reproduction rates (Frederickson *et al*. 1994) which obviously represents a direct reduction in the overall rate of worm biomass increase. Obviously this has an effect on the overall rate of stabilisation and processing, particularly since it has been demonstrated that the enhanced waste stabilisation achieved under worm treatment is only attained under conditions of high resident worm biomass (Frederickson *et al*. 1994). It seems reasonable to suggest, then, that to maximise the effectiveness of the combined treatment approach, the initial composting period should be no longer than the minimum necessary to bring about pathogen control of the input biowaste. Though this represents the kind of compromise balancing act so typical of much of environmental biotechnology, it is one which holds much promise. The combination of annelidic conversion with composting permits both enhanced stabilisation rate and product quality, with the additional bonus that the volatile organic content is also significantly reduced. It is also possible that the natural ability of worms to accumulate various hazardous substances within their bodies will also have implications for waste treatment, particularly if it proves possible to use them deliberately to strip out particular contaminating chemicals.

Annelidic conversion is currently very clearly a minority technology in this role, but the potential remains for it to play a role in the future biological treatment of waste, either as a standalone or, as seems more likely, as part of an integrated suite of linked processes. This seems particularly likely if the characteristically superior product derived from this process can be shown to be consistent, since specialist materials in the horticultural and gardening market generally tend to offer better returns. However, only time will tell whether this will prove to be sufficient financial incentive to offset the costs of production and encourage wider adoption of the technology.

Biowaste to ethanol

Around half of the total dry matter in plant origin biomass is cellulose, and since this makes up the majority of the biowaste component in MSW, it represents a huge potential source of renewable energy. As is widely appreciated, sugars can be broken down by certain micro-organisms to produce alcohols, of which ethanol (C_2H_5OH) is the most common. This is, of course, a well-known application for the production of alcoholic beverages across the world, typically using fermentative yeasts. These organisms are poisoned by ethanol accumulation

greater than about 10% which means that, in order to derive higher concentrations, approaches relying on distillation or fractionating are required. In a wider context, ethanol, either as the typical hydrated form (95% ethanol, 5% water) or as azeotropically produced anhydrate, makes a good fuel with excellent general combustion properties.

Historically, the realisation of the huge energy source locked up in the sugars of the cellulose molecule has always been a practical impossibility. The combination of the β 1–4 linkage in cellulose itself, coupled with its typically close association with lignin, making large-scale hydrolysis to sugars a costly and difficult prospect. Some early attempts employed enzymes from wood-rotting fungi working on a feedstock of old newspapers or pulp, though the energy involved in actually making the process work often became a limiting factor. In the mid-1990s, various researchers began to investigate the potential of genetically modified bacteria, by inserting appropriate sequences from a variety of naturally occurring wood-rotting organisms. In the following years, a number of technologies have emerged, based both on whole-organism and isolated-enzyme techniques, and the commercial processing of cellulose to alcohol now appears to be about to become a mainstream reality.

A number of countries have begun to show an interest in the potential gains to be had from developing a biowaste-based ethanol industry. Within the USA, many individual states have started to undertake feasibility studies for their own areas. A recent California Energy Commission Report, for example, has established that the state-wide annual generation of biowaste exceeds 51 million dry tonnes, comprising forestry residue, MSW and agricultural waste. The same document estimates the resultant maximum ethanol yield at more than 3 billion gallons (US).

There are several thriving biomass-to-ethanol production plants elsewhere in the USA and the world, though most of these make their alcohol from primary crop plants, rather than biowaste. As with biogas, further discussion of the wider aspects of ethanol and the role of biotechnology in energy production appears in a later chapter.

Eutrophic fermentation (EF)

Eutrophic fermentation was the name given by one of the authors to the experimental, wet, in-vessel, aerobically enhanced biodegradation process he designed to investigate accelerated decomposition, principally as an alternative to anaerobic digestion. *Fermentation* in this context relates to the wider application of the word to encompass all microbial breakdown and not simply the anaerobic production of alcohol and *eutrophic* refers to the nutrient-rich environment within which it takes place.

The process arose as a result of research into the enhanced aerated remediation of post-anaerobic digestion liquor. The idea of introducing air into liquid or slurrified waste, as was discussed in Chapter 6, is well established as a means

of treatment. A laboratory prototype treatment was developed for the specific effluent and was successfully trailed at pilot scale, before subsequently being further developed and extended to the treatment of biowaste.

Eutrophic fermentation involves rendering the waste into a fine slurry, which is then contained within a bioreactor (Figure 8.4), aerated by means of bubble diffusers at the base of the vessel, mixed and heated to around 35 °C. Within this environment, the input waste breaks down to leave only about 10% of the original volume as recoverable solids within 35 days. The process liquor itself is characteristically less strong than for anaerobic digestion and typically contains only some 6–10% suspended solids. Analysis of this liquid by the Agricultural Development Advisory Service (ADAS) has indicated that it has some potential fertiliser use, based on key performance indicators such as nitrogen, phosphorous and potassium levels, electrical conductivity, generalised nutrient content and heavy metal residues. The results are shown in full in Tables 8.4 and 8.5.

With a 'satisfactory' pH of 6, a 'low' BOD of 2790 mg/l, low ammoniacal nitrogen and the likely slow release of much of the total nitrogen content over

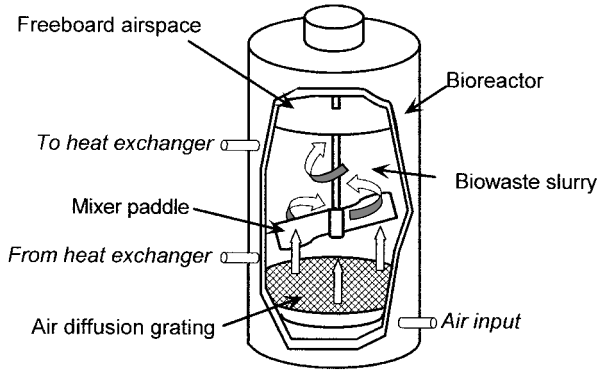

Figure 8.4 *EF bioreactor*

Table 8.4 *EF analysis results (nutrients)*

Principal nutrients	Units per 1000 gallons*	Comments
Nitrogen (ammoniacal)	2	Low
Nitrogen	5	Moderate
Magnesium	<1	Low
Phosphorus	1	Low
Potassium	6	Moderate

*Standard UK agricultural measure for fertiliser value.

Table 8.5 EF analysis results (metals)

Metal	mg/l	Comments
Cadmium	<0.25	Low
Chromium	<1	Low
Copper	<1	Low
Lead	<1	Low
Mercury	<0.01	Low
Nickel	<1	Low
Zinc	2.7	Low

several months, ADAS concluded that the liquor was a 'useful source of nitrogen and potash for crop growth'.

Though this method proved itself remarkably efficient in laboratory and intermediate trials, the company for which it was developed subsequently abandoned further research into biological waste treatment and, consequently, EF has never been taken to pilot scale. As a result, it is not possible to say how well it might perform, though for such aerobic processes utilising a completely mixed, suspended growth regime, even relatively small-scale studies tend to be fair representations of full-scale performance. This is particularly relevant to this system, as the hydrolysed organics are directly assimilated by the resident microbes, rather than simply accumulating in the process liquor.

In the foreseeable future, it is unlikely that this approach will make the transition to commercial application, not least as a result of outstanding intellectual property questions. Nevertheless, it stands as a worthwhile example of how alternative technologies can be developed to meet particular needs.

The issue of local circumstance is a recurrent theme throughout the whole of environmental biotechnology and probably so in waste management as much, if not more, than in any other aspect. As a consequence, it is highly unlikely that any one technology will establish itself as the monopoly treatment, which effectively also guarantees the scope for the further development of existing or novel minority biotechnologies.

Closing Remarks

Biowaste management cannot solely be about maximising the diversion of biodegradable material from landfill; it must seek to maximise the return of materials thus diverted back into the chain of utility as an equal priority. This is a particular consideration for those biological treatment processes that result in some kind of compost or similar product. Though it is beyond the scope of this book to discuss the issues of marketing and product quality in depth, it must be obvious that any significant level of biowaste diversion via these approaches demands

a sizeable and sustainable outlet. Suffice it to say that any such marketing operation requires significant assurances to the customer in terms of the material's quality, safety and value and moves are afoot to establish more widely agreed and accepted criteria. To this end, developments like the application of specific oxygen uptake rate (SOUR) testing as a means of objectively assessing microbial activity within the composting matrix may have a wider role to play.

Another area of concern often expressed is that of pathogen persistence, which leads many to view the need for sanitisation as synonymous with sterilisation, which, of course, it is not. The health risks potentially associated with biowaste processing to both workers and end-users has been well documented elsewhere and it is not our intention to restate that work here. However, what is less widely appreciated, and is more directly relevant to our central theme, is that particularly for large-scale applications a balanced and thriving community of micro-organisms is one of the most valuable contributions a good biowaste-derived soil amendment can make to poor soils. Removing pathogens, weed seeds and spores from biologically processed waste, while not producing a sterile wasteland, remains one of the key balancing acts of biowaste treatment.

Ironically, it is the expansion of biotechnology in areas beyond the development of better systems for immediate biowaste treatment which is likely to have major implications for waste management. For any processing technology, there is clear advantage in having a relatively pure input material and this has led to much discussion over the years regarding the respective benefits of separation on site against householder segregation. The latter approach has itself led to the plastic bags in which the biowaste so segregated becoming something of a nuisance at central treatment facilities, typically needing to be opened and removed, which is a labour-intensive operation at such a large scale. The increasing use of truly biodegradable plastics has already started to have an impact on the situation, especially at composting plants, since bags which will themselves decompose significantly reduce the amount of work involved.

If the predicted widespread use of cheap bioplastics, grown as alternative crops in transgenic plants, becomes a reality in the future, then this may itself have repercussions for the amount of material requiring biological treatment. Plastics account for around 8–10% of the developed world's waste stream and while reducing the demand for finite oil resources for the production of polymers has much to recommend it, one inevitable consequence will be to increase the amount of expressly biodegradable material in refuse. With growing numbers of countries looking to increase biowaste diversion, even allowing for effective recycling initiatives and attempts at waste minimisation, biotechnology may play an even larger part in the approaches to integrated waste management of the future.

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Case Study 8.1 Integrated Waste Management (Zurich, Switzerland)

As a method of waste management, anaerobic digestion has much to offer, especially considering its potential for integration into strategies designed to address wider environmental issues. The Otelfingen Kompogas plant, near Zurich airport, Switzerland, provides an excellent example.

Constructed in 1997, the half-hectare ($1\frac{1}{4}$ acre) facility processes around 14 000 tonnes of source separated biowaste annually, of which some 70% is domestic, the remainder being generated by commercial activities such as agriculture and food processing. Incoming waste is shredded and screened to remove any residual contaminants before being mixed with water to produce a slurry of 28% solids content for digestion. The bioreactor is of thermophilic plug flow design and has a retention period of 14–21 days. After processing, the digestate is dewatered by means of a screw press and the solid component, which is typically well within the relevant local heavy metal limits, is taken offsite and applied to farmland. Some of the water element is returned for reuse in the plant, while the excess is a valuable fertiliser substitute.

The biogas arising is of relatively high quality, being around 60% methane, and this energy source is exploited in two ways. Firstly, onsite engines are used to generate heat and electricity, which both covers the needs of the plant and produces a sizeable surplus for sale. Secondly, the company has invested in technology to clean up the gas to use as a fuel for its vehicle fleet, contributing a significant cost saving to its operational budget.

As a final example of biological integration, and of public and private sector cooperation, 50% funding from the Swiss government enabled the establishment of aquaculture greenhouses alongside the facility, where attenuated digester effluent is used to grow a variety of aquatic macrophytes.

Case Study 8.2 Co-composting for Reclamation (Colorado, USA)

The main advantages of composting as a means of biological waste treatment lie in its applicability to a wide range of materials and the potentially useful product which arises.

The reclamation of the Climax Molybdenum Mine, Leadville, Colorado required the revegetation of nearly 1600 hectares (4000 acres) of disturbed land, typically covered in tailings–the waste rock residue left after ore extraction. This was far from a straightforward task, since not only does such material provide a very poor footing for plants in the wind-buffered slopes, but also the growing season is only six or eight weeks long, the average temperature is slightly above 0 °C and nearly 7 metres (23 feet) of snow falls yearly.

The approach employed to overcome these difficulties is a good example of both the versatility of composting and the ability of biotechnology to provide a contemporary and integrated solution to more than one environmental problem. The burgeoning population and rapid growth in the resort towns of Summit County caused major strain on the existing arrangements for the treatment of sewage

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biosolids, driving up disposal costs. At the same time, ski trail expansion, and the increased construction work, generated enormous amounts of wood waste. After a good deal of negotiation, a cooperative venture was launched between the mine's owners, local sanitation facilities, land clearance firms, construction companies and haulers to produce a suitable compost out of the abundantly available materials.

Given the climatic conditions prevailing on the site, some 3300 metres (11 000 feet) above sea level, outdoor composting would seem an unlikely option, but considering the scale of the operation, highly engineered in-vessel systems would have been too expensive. By constructing oversized windrows to conserve heat and utilising effective control of aeration and careful blending of the input materials, it has proved possible to make good quality compost while also providing considerable economic benefits to all the parties involved.

To date, three years after it began, only around 40 hectares (100 acres) of the site has been reclaimed, so the project must, inevitably, be regarded as a very long-term one. However, the success of this combined biological treatment approach has already begun to stimulate great interest in both composting and reclamation in other areas with similar problems to solve.

9

Genetic Manipulation

Genes have been manipulated by man for a very long time, that is if selective breeding, which has been practised for centuries in agriculture and elsewhere to develop desirable characteristics in domesticated animals and plants, is to be considered as manipulation, as it rightly should. Even from the early days of Gregor Mendel, the Moravian monk and pioneer of genetic analysis, plants were bred to bring out interesting, useful and sometimes unusual traits. Many of these are now lost to classical plant breeders because of divergence of strains leading to infertile hybrids. One of the joys of genetic engineering is that in some cases, ancient genes may be rescued from seed found in archaeological digs for example, and reintroduced by transfer into modern strains. It has been proposed that the exchange of genetic information between organisms in nature is considerably more commonplace than is generally imagined (Reanney 1976) and could explain the observed rates of evolution. In bacteria, the most likely candidates for genetic transfer are plasmids and bacteriophage, and since eukaryotes lack plasmids, their most plausible vectors are eukaryotic viruses. This, of course, is in addition to DNA transfer during sexual reproduction. Current knowledge would suggest that exchange involving a vector requires compatibility between the organism donating the genetic material, the vector involved, and the recipient organism. For example, two bacteria must be able to mate for plasmid transfer to take place, or if a virus is involved as a vector, it must be able to infect both the donor and recipient cells or organisms. However, there is evidence to suggest that this view is somewhat naïve and that there is considerably more opportunity for genetic exchange between all cells, prokaryotic and eukaryotic, than is popularly recognised. This idea, proposed by Reanney (1976) is developed in Chapter 3.

Bacteria are notorious for their ability to transfer genes between each other as the need arises thanks to the location on plasmids of most of the gene groups, or operons, involved in the breakdown of organic molecules. Strong evidence for the enormous extent of these ‘genomic pools’ comes from analysis of marine sediment (Cook *et al*. 2001). Throughout this book, the point has been made that micro-organisms involved in remediation do so in their ‘natural’ state largely because they are indigenous at the site of the contamination and have developed suitable capabilities without any external interference. However, sometimes after a sudden contamination such as a spill, microbes are not able to amass useful mutations to their DNA quickly enough to evolve suitable pathways to improve

their fitness for that changed environment, and so they may be 'trained' by the artificially accelerated expansion of pre-existing pathways. The final option is that they may be genetically engineered. Organisms which represent the 'norm', frequently being the most abundant members occurring in nature, are described as 'wild type'. Those with DNA which differs from this, are described as mutant. Alteration can be by the normal processes of evolution which constantly produces mutants, a process which may be accelerated artificially, or by deliberate reconstruction of the genome, often by the introduction of a gene novel to that organism. This latter route is the basis of genetic engineering (GE) which has several advantages over traditional breeding or selection techniques. The process is specific, in that one gene, or a selected group of genes, is transferred and so the mutation is quite precise. There is flexibility in the system in that, depending on the modifications made to the genome, a new product may be produced or the level of expression of the existing product or products may be altered in quantity or proportions to each other. Another advantage often quoted is that GE allows genes to be transferred between totally unrelated organisms. The preceding discussion suggests that this is not a phenomenon unique to GE, but it is at least defined and specific.

Training: Manipulation of Bacteria Without Genetic Engineering

A general procedure is to take a sample of bacteria from, at, or near, the site of contamination from which a pure culture is obtained in the laboratory and identified, using standard microbiology techniques. The 'training' may be required either to improve the bacterium's tolerance to the pollutant or to increase the capabilities of pathways already existing in the bacterium to include the ability to degrade the pollutant, or a combination of both. Tolerance may be increased by culturing in growth medium containing increasing concentrations of the pollutant so that, over successive generations, the microbe becomes more able to withstand the toxic effects of the contaminant. Reintroduction of these bacteria to the polluted site should give them an advantage over the indigenous bacteria as they would be better suited to survive and remediate the contamination. Improving the microbe's ability to degrade a contaminant, sometimes referred to as catabolic expansion, may be increased by culturing the bacteria in growth medium in which the contaminant supplies an essential part of the nutrition, such as being the only carbon source. Only bacteria which have undergone a mutation enabling them to utilise this food source will be able to survive and so the method effectively selects for the desired microbe; everything else having died.

It has been argued that under laboratory conditions where cultures of bacteria are isolated from each other to prevent cross-contamination, mutations are most likely to occur as a result of an error in DNA replication. This is far less likely to be the most prominent source of mutation in nature, as the microbes are constantly in close proximity with other organisms and, consequently, the opportunity for

exchange of genetic material is enormous. In fact the process of DNA replication has a very high fidelity, the reasons for which are obvious. An increased rate of error may be forced upon the organism, speeding up the rate of mutation, by including a mutagen in the growth medium. A mutagen is a chemical which increases the rate of error in DNA replication, often by causing a very limited amount of damage to the DNA such that the DNA polymerase, the enzyme responsible for synthesising DNA, is unable to determine the correct base to add in to the growing nucleotide chain. If the error in the nascent strand cannot be recognised and corrected, the fault becomes permanent and is handed on through the generations.

Manipulation of Bacteria by Genetic Engineering

Genetic manipulation by the deliberate introduction of defined genes into a specified organism is a very powerful technique which is relatively new and certainly in constant development, sometimes at phenomenal rates of progress. The techniques have produced some exciting hybrids in all areas of research, both microscopic; bacteria and fungi, usually described as recombinants, and macroscopic; principally higher plants and animals, commonly described as transgenics. The latter term refers to the principle of deliberate transfer of a gene from one organism to another in which it is not normally resident. This earns the incoming gene the title of 'foreign'. Some examples of these which are relevant to environmental biotechnology will be discussed later in this chapter.

Some of the developments are of great potential interest and represent some exciting and innovative work. However, it must be said that, in practice, a very tiny proportion of all endeavour in the name of environmental biotechnology has, or is likely to have in the future, a direct reliance for its effectiveness on the type of recombinants and transgenics currently being developed. This is not because of the limits of genetic engineering, which in principle are almost boundless, given sufficient resources, but because of cost. It is a principal factor as the technology and research to produce transgenic organisms attract an inherently high price. While such a situation may be sustainable by pharmaceutical companies and perhaps to a lesser extent, agribiotechnology companies possibly able to command a high return on sales of the product, it is rarely sustainable in applications of environmental technology. Few commercial organisations are excited at the prospect of spending a large proportion of their income on waste disposal for example, and will normally only do so when absolutely necessary.

There are other factors which affect the suitability of transgenic organisms in this science due to current requirements for containment. In addition, the way in which such a recombinant is utilised may cause problems of its own. For example, if the recombinant is a micro-organism structured to improve the rate of degradation of a pollutant, its performance may be exemplary in laboratory conditions but when it is applied in bio-augmentation it is in competition with

indigenous species which could outgrow the recombinant. The novel bacterium may also lose its carefully engineered new capability through normal transfer of genes given the high level of promiscuity between bacteria. A highly controlled and contained environment such as a bioreactor may circumvent some of these objections but it is not always practical to move the contamination to the solution rather than the solution to the contamination. Again this involves expense and practical considerations, not least of which are safety concerns associated with the transport of contaminated material.

In reality, there is rarely any need to use recombinants or transgenics and it is far more likely that the required metabolic capability will be provided by indigenous organisms, or ones which have been trained for the task. There are, however, some exotic and ingenious applications, and by way of illustration, some examples are given here. The aim is to provide an overview of some of the more frequently used technologies together with specific examples. There are very many excellent textbooks and specialised publications which should be consulted should a more detailed and working knowledge be required. However, an overview of the principles of genetic engineering are given here for the benefit of those unfamiliar with the technology.

Basic Principles of Genetic Engineering

There are endless permutations of the basic cloning procedures but they all share some fundamental requirements. These are: the enzymes, solutions and equipment necessary to perform the procedures; the desired piece of DNA to be transferred; a cloning vector; and the recipient cell which may be a whole organism. For the process to be of any measurable value, it is also essential to have some means of determining whether or not the transfer has been successful. This is achieved by the use of marker genes. The requirements referred to above are described in the following sections.

Enzymes, solutions and equipment

There are many steps involved in the isolation of DNA which now have become standard laboratory techniques. Once DNA has been isolated from an organism, it is purified from contaminating material such as protein and is precipitated out of aqueous solution by the addition of alcohol, for example ethanol, to approximately 70%. The DNA appears as a white, semi-transparent material, coiling out of solution on addition of the alcohol. This may be collected by centrifugation and dried down ready for the next stage which is usually enzyme digestion. The aim of the next stage is to insert the DNA into the vector, for which the ends of the DNA and the vector have to be prepared. This may be done by restriction endonucleases which recognise specific sequences within the DNA and cut at that site, either producing a flush or staggered end, Figure 9.1, or by incubation

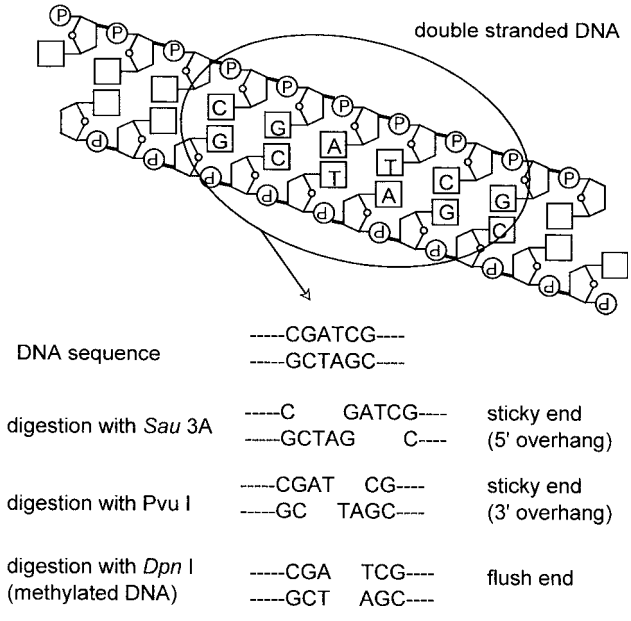

Figure 9.1 *Restriction enzymes*

over a very limited time period with an exonuclease which digests the end of the DNA and followed by further digestion with another nuclease which tidies up the ends to produce flush ends. There are other restriction nucleases which recognise a site in DNA but cut at some distance from it, but these are rarely of any value in cloning procedures.

Preparation of the vector is dictated by the type of end prepared for the insert DNA: flush or 'sticky'. If it is flush, it does not much matter how that was achieved so long as the vector receiving it is also flush, but if it is sticky, the appropriate sticky end must be prepared on the vector by a suitable restriction endonuclease. There are many methods of DNA and vector preparation all of which have their advantages and disadvantages and, although interesting in themselves, are beyond the scope of this book.

Having prepared the ends, the next step is to stick the pieces together. The prepared insert, or 'foreign' DNA is incubated with the prepared vector in an aqueous solution containing various salts required by the enzymes, and ligase which is an enzyme, the function of which is to make the bond between the free phosphate on a nucleotide base and the neighbouring ribose sugar, thus 'repairing' the DNA to make a complete covalently linked chain. This recombinant DNA molecule may be transferred into a cell where it undergoes replication in the usual way. If the DNA is not viral, introduction will be by direct entry through the cell membrane achieved by any one of a number of standard techniques all

based on making the membrane permeable to the DNA molecule. However, if the 'foreign' DNA is part of a recombinant virus, it has to be packaged into particles, and then transferred into cells by infection. A check may be made on the product by carrying out analyses described later.

DNA for transfer

Most commonly, this is a piece of double-stranded DNA which contains the coding sequence for a gene. It may have been obtained from a number of sources, for example, genomic DNA, a cDNA library, a product of a polymerase chain reaction (PCR) or a piece of DNA chemically produced on a DNA synthesiser machine. Another source is from a DNA copy of an RNA virus as in the replicative form of RNA viruses.

Genomic libraries

Genomic DNA, in this context, is material which has been isolated directly from an organism, purified and cut up into pieces of a size suitable to be inserted into a cloning vector. These pieces may either be ligated in total mixture, into a suitable vector to produce a genomic library, or a specific piece may be isolated and prepared as described above. Genomic libraries are very useful, as they may be amplified, and accessed almost limitlessly, to look for a specific DNA sequence thus reducing the amount of work involved in any one experiment. The disadvantage is that if the genomic library is of a eukaryotic origin, which is almost exclusively the case, the genes will contain regions, or introns, which are quite normally inserted along its length and are processed out of the RNA copy during maturation prior to protein synthesis. This is a problem if the gene is to be expressed, in other words, if the protein is to be made from the DNA blueprint. Prokaryotes do not contain introns in their genes and so do not possess the mechanisms for their removal. Furthermore, introns are not necessarily processed correctly even if the expression system is eukaryotic. This problem can be avoided by using a cDNA instead of a genomic library.

cDNA libraries

In eukaryotes, the first product of transcription from DNA is not messenger RNA (mRNA) but heterogeneous nuclear RNA (hnRNA). This is mRNA prior to the removal of all the noncoding sections, or introns, which are discarded during the processing to produce the mature mRNA. Complementary DNA (cDNA) is DNA which has been artificially made using the mature mRNA as a template, which is then used as the template for the second strand. Thus the synthetic DNA product is simply a DNA version of the mRNA and so should overcome the problems of expression outlined above.

Polymerase chain reaction

The polymerase chain reaction (PCR) is a powerful technique which amplifies a piece of DNA of which only a very few copies are available. The piece must be

flanked by DNA whose sequence is known or at least a close approximation can be guessed. This knowledge allows a short sequence of DNA to be synthesised of only a few nucleotides long, to bind specifically to the end of the sequence and act as a primer for the DNA polymerase to make one copy of the whole piece of DNA. A second probe is used for the other end to allow the second strand to be synthesised. The process is repeated by a constant cycling of denaturation of double-stranded DNA at elevated temperature to approximately 95 °C, followed by cooling to approximately 60 °C to allow annealing of the probe and complementary strand synthesis. This technique requires the use of DNA polymerases able to withstand such treatment and two bacteria from which polymerases have been isolated for this purpose are *Thermococcus litoralis* and *Thermus aquaticus*. This latter extremophile has been discussed in Chapter 3.

Cloning vectors

A cloning vector is frequently a plasmid or a bacteriophage (bacterial virus) which must be fairly small and fully sequenced, able to replicate itself when reintroduced into a host cell, thus producing large amounts of the recombinant DNA for further manipulation. Also it must carry on it 'selector marker' genes. These are different from the reporter genes described below which are indicators of genomic integrity and activity. A common design of a cloning vector is one which carries two genes coding for antibiotic resistance. The 'foreign' gene is inserted within one of the genes so that it is no longer functional therefore it is possible to discriminate by standard microbiology techniques which bacteria are carrying plasmids containing recombinant DNA and which are not. Selector genes may operate on at least two levels, the first at the level of the bacterium, usually *Eschericia coli*, in which the manipulations are being performed described above and the second being at the level of the final product, for example, a higher plant. In this case such a selector gene can be resistance to antibiotics like kanamycin or hygromycin.

Standard cloning vectors normally carry only selector marker genes required for plasmid construction. To make the manipulations easier, these genes normally contain a multicloning site (MCS) which is a cluster of sites for restriction enzymes constructed in such a way to preserve the function of the gene. Disruption by cloning into any one of these sites will lose the function of that gene and hence, for example, if it codes for antibiotic resistance, will no longer protect the bacterium from that antibiotic. An example is shown in Figure 9.2. This is pGEM® (Promega 1996) which has a MCS in the ß-*gal* gene. This codes for ß-*galactosidase* from the *E. coli lac* operon, which has the capacity to hydrolyse x-gal, a colourless liquid, to produce free galactose and 'x' which results in a blue pigment to the colony. Thus the screening for successful insertion into the MCS is a simple scoring of blue (negative) or white (possibly positive) colonies. The success of the experiment can be determined quickly as this cloning vector also has sequences at either side of the MCS which allows for rapid DNA sequencing.

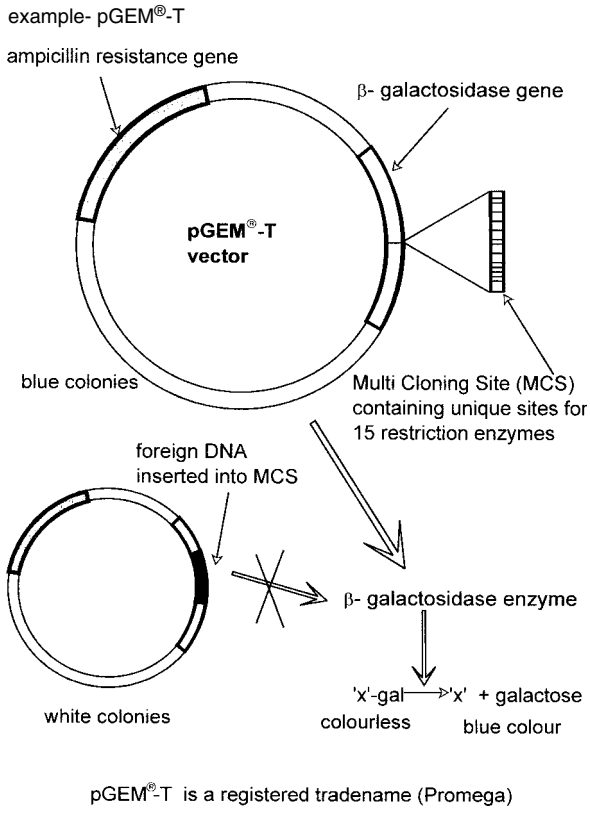

Figure 9.2 *Cloning vector*

Additionally, some eukaryotic viruses may be used as vectors but these tend to be so large that direct cloning into them is difficult. A solution to this is to carry out manipulations on the desired DNA fragment cloned into a bacterial plasmid and then transfer the engineered piece into the virus thus making a recombinant eukaryotic virus. One such virus now used extensively in genetic engineering, both as a cloning vehicle and as an excellent expression vector, is Baculovirus illustrated in Figure 9.3 which is also effective as a bioinsecticide.

Expression vectors

These are similar to the vectors described above but in addition have the required signals located before and after the 'foreign' gene which direct the host cell to translate the product of transcription into a protein. It is sometimes a difficult, expensive or time-consuming procedure to analyse for product from the 'foreign' gene and so, in addition to the selector genes described above, there are frequently

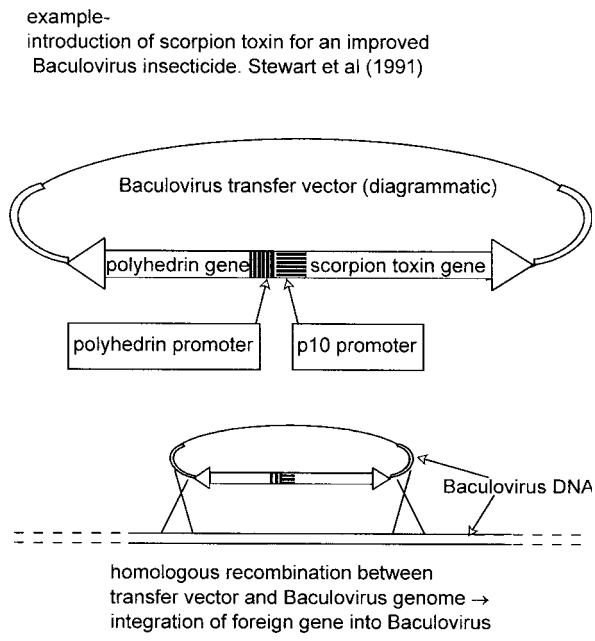

Figure 9.3 *Recombinant Baculovirus*

reporter genes to indicate whether or not the signals are 'switched on' allowing the 'foreign' DNA to be expressed. There are many reasons which are difficult to predict, why even a perfectly constructed gene may not be functional, such as the consequence of the exact site of insertion in the genome; hence the need for inbuilt controls.

Reporter genes

There are many such genes in common use and these usually code for an enzyme. The most common is *β-galactosidase*, mentioned above. This enzyme, supplied with the appropriate reagents, may also catalyse a colour change by its activity on a variety of chemical compounds typified by orthonitrophenolgalactoside (ONPG) which changes from colourless to yellow on hydrolysis in much the same way as the blue/white screening described above for the cloning vector, pGEM®. Other reporter genes produce enzymes which can cause the emission of light such as the *luciferase* isolated from fireflies, or whose activity is easy and quick to assay like the bacterial β-glucuronidase (GUS), which is probably the most frequently used reporter gene in transgenic plants. Reporter genes can only be a guide to the process of transcription and translation occurring in the cell and it has been acknowledged for some time that care must be exercised to avoid misinterpreting data (Pessi, Blumer and Haas. 2002).

As with selector genes, the reporter genes serve no useful purpose once the cloning procedure has been successfully accomplished to produce the finished product. In the early days of this technology, these genes would normally be left *in situ* to avoid the extra work of removing them which might also upset the structure of the recombinant genome thus diminishing the quality of the carefully engineered organism. There is, however, an argument to remove all genes which were necessary for construction purposes but which no longer serve a useful purpose, to reduce perceived potential risk of unwittingly increasing the spread of genes throughout the environment. These concerns are addressed in Chapter 11.

Analysis of Recombinants

The design of the plasmid was such that insertion of 'foreign' DNA allows for a colour test, or causes a change in antibiotic sensitivity, either to resistance (positive selection) or sensitivity (negative selection). This constitutes the first step in screening. The second stage is usually to probe for the desired gene using molecules which will recognise it and to which is attached some sort of tag, usually radioactive or one able to produce a colour change. The next stage is normally to analyse the DNA isolated from possible recombinants, firstly by checking the size of the molecule or pieces thereof, or by sequencing the DNA. This is the most informative approach but used to be very laborious. With the current and ever-developing automated protocols, DNA sequencing has become a standard part of recombinant analysis procedure. However, if a large number of samples are to be analysed it is usually quicker and cheaper to scan them by a procedure described as a Southern blot, after Ed Southern, the scientist who designed the technique.

In this procedure, the DNA is spread out by electrophoresis on a gel which is then probed by a piece of radioactive DNA complementary to the sequence of interest. If a band shows up on autoradiography then the probe has found a mate and the required sequences are present, at least in part. DNA sequencing is then required to confirm exactly what has occurred during the cloning procedure but the advantage is that only the samples which are very likely to contain the required insert are sequenced, thus saving time and expense.

From this technique, has developed the Northern blot which is much the same idea except that the material spread out on the gel is RNA rather than DNA, and the Western blot which is slightly different in that the material electrophoresed is protein, which is probed with antibodies against the anticipated protein, rather than nucleic acid, as is the case in Southern and Northern blots.

Recombinant Bacteria

Genetic engineering of micro-organisms for use in environmental biotechnology has tended to focus on the expansion of metabolic pathways either to modify

the existent metabolic capability or to introduce new pathways. This has various applications, from the improved degradation of contaminants, to the production of enzymes for industry, thus making a process less damaging to the environment. One such experimental example taken from 'clean technology' with potential for the manufacturing industry, is a strain of *Eschericia coli* into which was engineered some 15 genes originating from *Pseudomonas*. These were introduced to construct a pathway able to produce indigo for the dyeing of denim, commented on by Bialy (1997). The traditional method requires the use of toxic chemicals with the associated safety measures and inherent pollution problems. Similar technologies were investigated in the early 1980s, by Amgen in the USA and Zeneca in the UK, but were not pursued due to questionable profitability. Whether or not this route will now be taken up by industry remains to be seen (BMB 1995).

Recombinant Yeast

Yeast, being unicellular eukaryotes, has become popular for cloning and expressing eukaryotic genes. These are fairly simple to propagate, some species being amenable to culture in much the same way as bacteria. Yeast cells are surrounded by a thick cell wall which must be removed to permit entry of DNA into the cell. There are several types of plasmid vector available for genetic engineering, some of which have been constructed to allow replication in both bacteria and yeast (Beggs 1981). All have a region which permits integration into the host yeast genome by recombination. This occurs by alignment of the sequences complementary between the host genome and the incoming plasmid DNA. Two crossover events then take place which effectively swap over a piece of host DNA with the plasmid DNA. A similar process occurs in the construction of recombinant Baculoviruses.

Recombinant Viruses

The insect virus, Baculovirus, has been shown to be the method of choice for the overexpression of genes in many applications of molecular biology. The viral genome is large relative to bacterial plasmids and so DNA manipulations are normally carried out on a plasmid maintained in *Eschericia coli*. Introduction of the reconstructed gene, or group of genes, to the Baculovirus DNA occurs by recombination in much the same way as described for the formation of recombinant yeast. One example of interest to environmental biotechnology is the replacement of p10, one of the two major Baculovirus proteins, polyhedrin being the other, by the gene for a scorpion neurotoxin, with a view to improving the insecticidal qualities of the virus, sketched in Figure 9.3 (Stewart *et al.* 1991). The 'promoters' at the start of the gene, referred to in the figure, are the regions of RNA which regulate protein synthesis, from none at all, to maximum expression.

Transgenic Plants

Currently, genetic engineering in agribiotechnology is focusing on genetic modifications to improve crop plants with respect to quality, nutritional value, and resistance to damage by pests and diseases. Other avenues under investigation aim to increase tolerance to extreme environmental conditions, to make plants better suited for their role in pollutant assimilation, degradation or dispersion by phytoremediation, or to modify plants to produce materials which lead to the reduction of environmental pollution. Crop quality improvements such as the control of fruit ripening (Grierson and Schuch 1993), an example of which is the oft quoted, Flavr-Savr tomato, and the production of cereals with improved nutritional value, are not addressed here because, although of great interest to the food industry, are of more peripheral relevance to environmental biotechnology. Many of the transgenic plants, examples of which are given later in this chapter, have been produced using the Ti plasmid transfer system of *Agrobacterium tumefaciens* and often, together with the 35S CaMV promoter. Both of these tools are described from a GE technique viewpoint in this chapter and from a biological standpoint in Chapter 10.

Transformation of plants

There are two practical problems associated with genetic engineering of plants which make them more difficult to manipulate than bacteria. Firstly they have rigid cell walls and secondly they lack the plasmids which simplify so much of genetic engineering in prokaryotes. The first problem is overcome by the use of specialised techniques for transformation, and the second by performing all the manipulations in bacteria and then transferring the final product into the plant. The DNA construct contains regions of DNA which are complementary to the plant DNA to enable the inserted piece to recombine into the plant genome.

The most popular method of transforming plants is by the Ti plasmid but there are at least two other methods also in use. The first is a direct method where DNA is affixed to microscopic bullets which are fired directly into plant tissue. An example of this technology is the introduction into sugarcane, of genes able to inactivate toxins produced by the bacterium, *Xanthomonas albilineans*, causing leaf scald disease (Zhang, Xu and Birch 1999). This method of biolistic bombardment, may increase in popularity in line with improvements to plastid transformation. It is now possible to produce fertile transgenics expressing foreign proteins in their edible fruit (Ruf *et al.* 2001).

The second is by protoplast fusion which is a process whereby the plant cell wall is removed leaving the cell surrounded only by the much more fragile membrane. This is made permeable to small fragments of DNA and then the cells allowed to recover and grow into plants. These methods can be unsuccessful due to difficulties in recovery of the cells from the rather traumatic treatments and also because the DNA introduced, has a tendency to be inserted randomly

into the genome, rather than at a defined site. However, both methods enjoy the advantage that DNA enters the cell exactly as constructed and has not passed through an intermediate vector giving the opportunity for gene rearrangement.

Transformation by the Ti plasmid of *Agrobacterium tumefaciens*, shown diagrammatically in Figure 9.4, suffers from few disadvantages other than the limitation that it does not readily infect some cereal crops. This potential problem has been addressed by attempting to increase its host range (Godwin, Fordlloyd and Newbury 1992) which has met with success, leading to improved transformation procedures (Le *et al*. 2001). In essence, the wild-type plasmid contains genes which causes the transfer of a piece of DNA, 'T-DNA', into a plant cell. This piece is bordered by sequences of 24 base pairs in length which are repeats of each other. This structure is fairly common in DNA and is described as direct repeat. The T-DNA comprises genes which cause crown gall disease. These

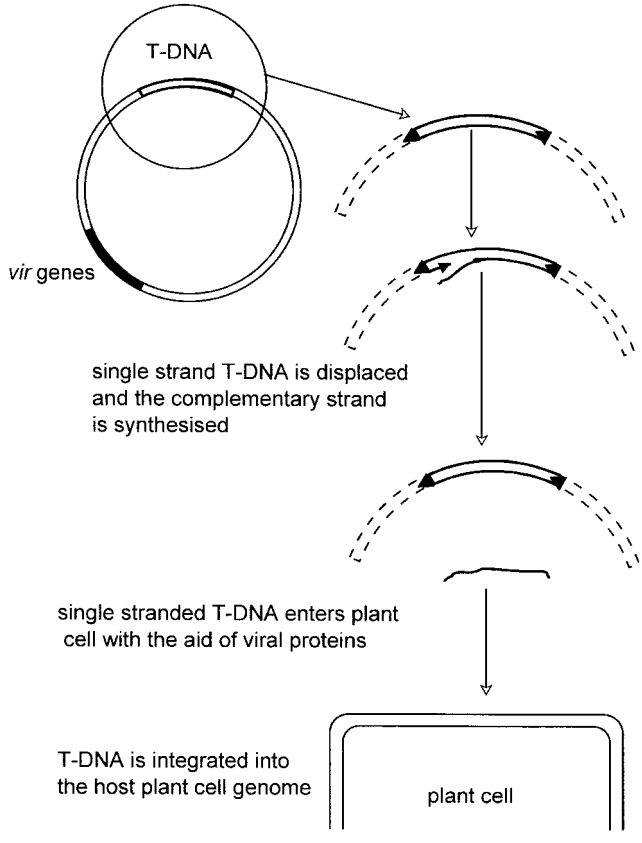

Figure 9.4 *Ti plasmid of Agrobacterium tumefaciens*

genes may be cut out and replaced by DNA containing the gene of choice to be introduced into the plant. There are many additional elements which may be included in the construct. For example, if the aim is to express the gene, it is preceded by a strong promoter, most commonly the '35S' promoter of Cauliflower Mosaic Virus (CaMV).

In addition to the above, it is important to know if the 'foreign' gene is being expressed and so frequently a 'reporter' gene described in the section above is also included located close to the gene of interest. Recombination is not 100% efficient, and so a method of selection is required such that only plants containing the novel DNA grow. This is frequently a gene coding for resistance to weedkiller or antibiotic. On the grounds of size, this is usually introduced more successfully on a second Ti plasmid during a co-infection with *Agrobacterium* carrying the plasmid containing the gene of interest. The experiment can become somewhat complicated at this stage, as other selector genes are introduced into the plasmids to ensure that growth is only possible if all the desired elements are present in the plant cell. This can involve infection with two or three cultures of *Agrobacterium* each containing its own engineered Ti plasmid. A very detailed description of the Ti plasmid is published elsewhere (Hughes 1996).

Examples of developments in plant GE

The purpose of these examples is to illustrate the potential plant genetic engineering could bring to future practical applications in the field of environmental biotechnology. In some cases the intention is to reduce the amount of herbicide and pesticides, or other agricultural chemicals required to produce a given crop yield, in others it is to improve tolerance of harsh conditions or to protect the plants from attack thus reducing wastage. The intention is to note the technical details here, while the effects such developments may have on the environment as a whole, feature elsewhere throughout this book.

Broad range protection

A general strategy to protect plants from various viruses, fungi and oxidative damage by a range of agents, has been proposed using tobacco plants as a model. The transgenics express the iron-binding protein, ferritin, in their cells which appears to afford them far-ranging protection (Deák *et al*. 1999).

Resistance to herbicides

'Glyphosate', one of the most widely used herbicides, is an analogue of phosphoenol pyruvate and shows herbicidal activity because it inhibits the enzyme *5-enolpyruvylshikimate-3-phosphate synthase*. The gene coding for this enzyme has been identified, isolated and inserted into a number of plants including

petunias. In this case, the gene was expressed behind a CaMV promoter and introduced using *A. tumefaciens*, leading to very high levels of enzyme expression. As a consequence, the recombinant plants showed significant resistance to the effects of glyphosate (Shah *et al.* 1986). Developments in this strategy include the formation of a chimaeric *synthase* enzyme, the analysis of which should lead to improved herbicide resistance in transgenic crops using this strategy (He 2001).

An alternative approach but still using *A. tumefaciens* has been to transfer the genes for mammalian cytochrome P450 monooxygenases, known to be involved in the detoxification (and activation) of many xenobiotics including pesticides, into tobacco plants. These transgenics displayed resistance to two herbicides, *chlortoluron* and *chlorsulphuron* (Yordanova, Gorinova and Atanassov 2001).

Improved resistance to pests

Plants have an inbuilt defence mechanism protecting them from attack by insects but the damage caused by the pests may still be sufficient to reduce the commercial potential of the crop. The usual procedure is to spray the crop with insecticides but in an effort to reduce the amount of chemical insecticides being used, plants are being engineered to have an increased self-defence against pests. Attack by insects not only causes damage to the plant but also provides a route for bacterial or fungal infection in addition to the role played in the spread of plant viruses. With a view to increasing resistance to sustained attack, the genes coding for the *δ-endotoxin* of the bacterium, *Bacillus thuringiensis* (Bt), described a little more fully in Chapter 10, have been transferred into plants. Examples are of synthetic *B. thuringiensis δ-endotoxin* genes transferred, in the first case, by *A. tumefaciens* into Chinese cabbage (Cho *et al.* 2001) and in the second, by biolistic bombardment into maize (Koziel *et al.* 1993). In both cases, the transgenic plants showed greatly improved resistance to pest infestation. There are, however, some problems with crop performance of some genetically engineered plants highlighted in Magg *et al.* (2001). Insects are able to develop resistance to Bt products which is a problem addressed by insertion of *δ-endotoxin* genes into the chloroplast genome rather than into that of the plant's nucleus, with promising early results (Kota *et al.* 1999).

It may be recalled, that for each amino acid incorporated into a protein there is usually a choice of three or four codons all of which code for that same amino acid. Different organisms have distinct preferences for a particular codon, thus *Bacillus thuringiensis* tends to use codons richer in thymidine and adenine than the plant cells into which the gene is placed. There are also signals controlling the expression of these genes relevant to bacteria, rather than eukaryotes, which will not function very well, if at all, in the plant cell. For these reasons, expression may benefit from modification of the DNA sequence to compensate for these differences while maintaining the information and instructions. This may account in part for the very high levels of expression and stability of the Bt proteins whose genes have been introduced, by (biolistic) microbombardment, into chloroplasts

(De Cosa *et al*. 2001) which, because of their prokaryotic ancestry, have 'protein synthesising machinery' more in keeping with prokaryotes than the eukaryotic cell in which they cohabit.

Attempts to improve virus resistance have led to the introduction, by *A. tumefaciens*, of the genes expressing antibodies to the coat protein of Tobacco Mosaic Virus (TMV). Expression of these in the plant led to complete immunity against TMV (Bajrovic *et al*. 2001).

Improved resistance to disease

Bacteria communicate with each other by way of small diffusible molecules such as the N-acylhomoserine lactones (AHLs) of Gram negative organisms. In this way, described as 'quorum sensing', they are able to detect when a critical minimum number of organisms is present, before reacting. These responses are diverse and include the exchange of plasmids and production of antibiotics and other biologically active molecules. Plants are susceptible to bacterial pathogens such as *Erwinia carotovora*, which produces enzymes capable of degrading its cell walls. The synthesis of these enzymes is under the control of AHLs and so they are made only once the appropriate threshold level of this chemical has been reached. The rationale behind using AHLs for plant protection is to make transgenic plants, tobacco in this case, which express this signal themselves. The consequent high level of AHL presented to the pathogenic bacteria, wrongly indicates a very high number of similar organisms in the vicinity, and triggers the bacteria into responding. As a consequence, they produce enzymes able to degrade the plant cell walls and continue infection. The plant will mount its normal response to invasion but on a far greater scale than necessary to destroy the few bacteria actually causing the infection, thus improving the plant's resistance to the disease. It seems complicated, but research into the validity of the hypothesis is under way (Fray *et al*. 1999).

Improved tolerance

Plant–microbe interactions are addressed in Chapter 10. Among the examples given are that of *Pseudomonas syringae* which colonises the surface of leaves. This example is of bacterial rather than plant modification but impinges on interaction between the two. *Pseudomonas syringae* produces a protein which promotes the formation of ice crystals just below 0 °C thus increasing the risk of frost damage. Lindow *et al*. (1989) have identified and isolated the gene for this protein. They transferred it to the bacterium *Eschericia coli* to simplify the genetic manipulations. This required the deletion of sufficient regions so that a truncated, and therefore nonfunctional, ice mediating protein was expressed. They reintroduced this mutated gene into *Pseudomonas syringae* and selected for ice^- mutants which were no longer able to produce the ice nucleating protein. Many such regimes fail in practice because it is difficult to maintain a population of

mutant bacteria in a community dominated by the wild type as, frequently, the latter will soon outnumber the mutant by competition for nutrients, since it is usually better adapted to the particular environment than the mutant. However, in this case, due to massive application of *Pseudomonas syringae ice*$^-$ to strawberry plants, the mutants were able to compete with the wild type and protect this particularly susceptible crop against frost damage.

Salt tolerance in tomatoes has been established by introducing genes involved in Na^+/H^+ antiport, the transport of sodium and hydrogen ions in opposite directions across a membrane. The quality of the fruit was maintained by virtue of the fact that the sodium accumulation caused by the antiport occurred in leaves only and not in the fruit (Zhang and Blumwald 2001).

Improved tolerance to drought, salt and freezing in *Arabidopsis* has been achieved by overexpressing a protein which induces the stress response genes. However, if too much of this factor is produced, which was the case when the 35S CaMV was employed, severe growth retardation was observed. No such problem existed when instead, the overexpression was under the control of a promoter which was only switched on when stressful conditions existed (Kasuga *et al*. 1999).

Improved plants for phytoremediation

Chapter 7 mentioned the genetic modification of a poplar to enable mercury to be removed from the soil and converted to a form able to be released to the atmosphere. This process is termed 'phytovolatilisation' (Rugh *et al*. 1998). The modification required a gene to be constructed, styled on the bacterial *mer A* gene, by making a copy reflecting the codon bias found in plants using PCR technique. The *mer A* gene is one of a cluster of genes involved in bacterial detoxification of mercury, and is the one coding for the enzyme, *mercuric ion reductase*, which converts mercury from an ionic to a volatile form. Initially the constructed *mer A* gene was expressed in *Arabidopsis thalia* (rape) where resistance to mercury was observed, and in this study, the gene was transferred by microprojectile bombardment ('gene guns') to poplar tree (*Liriodendrn tulipifera)* embryogenic material. When the resulting yellow poplar plantlets were allowed to develop, they were found to exhibit tolerance to mercury and to volatalise it at 10 times the rate observed in untransformed yellow poplar plantlets. This study demonstrated the possibility that trees can be modified to become useful tools in the detoxification of soil contaminated with mercury. These studies were pursued in *Arabidopsis thalia* where it was observed that successful remediation also required the *mer B* genes coding for a *lyase* (Bizily, Rugh and Meagher 2000).

A bacterial gene encoding *pentaerythritol tetranitrate reductase*, an enzyme involved in the degradation of explosives, has been transferred into tobacco plants. The transgenics have been shown to express the correct enzyme and trials are under way to determine their ability to degrade TNT (French *et al*. 1999).

Developments in the use of transgenic plants for bioremediation have been reviewed (Francova *et al*. 2001).

New products from plants

The rape plant, *Arabidopsis thalia* has become a popular choice for the production of recombinant species. One such recombinant is a rape plant, the fatty acid composition in the seed of which has been modified. It now produces triacylglycerols containing elevated levels of trierucinic acid suitable for use in the polymer industry (Brough *et al*. 1996) and, in a separate project, polyhydroxybutyrate suitable for the production of biodegradable plastics (Hanley, Slabas and Elborough 2000). Synthesis of the copolymer poly(3-hydroxybutyrate – *co* – 3-hydroxyvalerate) by *Arabidopsis*, is another example of the application of *Agrobacterium tumefaciens* technology and the use of the 35S promoter from Cauliflower Mosaic Virus (Slater *et al*. 1999). This copolymer can be produced by bacterial fermentation, but due to cost considerations, it is normally synthesised chemically. This example is discussed further in Chapter 10 under the umbrella of 'clean' technology. Progress in this field has been reviewed recently (Snell and Peoples 2002).

Closing Remarks

It is something of an irony that genetic engineering is virtually synonymous with biotechnology. The advances in this field have been enormous and, in many areas, are of singularly great importance, yet its impact has been much less dramatic when considering the purely environmental aspects. So much of what has been discussed in this chapter has not managed to make the wholesale transition into mainstream commercial activity and whether it ever will still remains to be seen. Unquestionably, GE will continue to play a definitive role in the future development of the biological sciences; its position in respect of practical environmental biotechnology is, perhaps, much less clear at this point.

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Case Study 9.1 Engineered Salt Tolerance (Rehovot, Israel)

One area where agrobiotechnological advances could have distinctly environmental applications, in the widest sense, is in the production of transgenic plants. While much of this research has centred on greater productivity, some work has been done to address other issues and one of the potentially most important of these relates to improving salt tolerance.

According to some authorities, over half the world's agricultural land is expected to become increasingly saline within the next 50 years. In some countries, such as Israel where this work was pioneered, fresh water supplies are already stretched to the point that farmers are forced to use a proportion of salty water for irrigation and it is anticipated that this usage will continue to grow in the future. If encroaching desertification is to be avoided under these circumstances, then the development of salt-resistant crops and trees becomes essential.

Researchers at the Hebrew University in Rehovot isolated the protein, *BspA*, which is produced by the European aspen, *Populus tremula*, when growing in salty conditions and appears to protect the tree's cells from damage by attracting water molecules, though the exact mechanism is still unknown. By introducing the appropriate genes into other plant species, it is hoped that the varieties produced may be afforded the same kind of protection.

Attempts to achieve this have been undertaken and the abilities of a related tree, the Euphrates poplar, *Populus euphratica*, which is even more salt-tolerant are also under investigation to see if it has other, additional methods which could likewise be incorporated into future generations of transgenic crops. If this work proves successful, it would herald a major breakthrough in mitigating a major cause of global environmental degradation.

10

Integrated Environmental Biotechnology

The essence of environmental biotechnology as an applied science, as we have set out to demonstrate in the preceding chapters of this book, is the harnessing of pre-existing organisms and natural cycles to bring about a desired goal. Sometimes this is achieved by relatively unsophisticated means. At others it requires rather more in the way of engineering, adaptation or modification, in one form or another, to fit nature's original to the intended purpose. Thus, though the exact form of any given iteration may differ, the underlying paradigm remains the same. Applying what is effectively a naturalistic model leads to some inevitable conclusions with far-reaching implications for the future of this particular discipline.

The fundamental necessity of mutual interactions in nature is readily accepted and understood. Hence, the natural cycles obligatorily dovetail together at both the gross and the microscopic levels, with interplay existing between the organism and its environment as well as between the various central metabolic pathways. Since such integration exists already between bioprocesses, and these are the very stuff upon which environmental biotechnology is based, the potential for integrated applications is clear.

At its simplest, this involves the sequential use of individual technologies to provide a solution in a linked chain of successive steps, often termed a 'treatment train'. The other extreme is the wider amalgamation of larger fundamental problems and their resolutions into a single cohesive whole. This book began by looking at the key intervention areas for environmental biotechnology and defined the three legs of that particular tripod as pollution, waste and manufacturing. This theme has been further developed, to examine how old pollution can be cleaned up and how the rational treatment of solid wastes and effluent can contribute to the reduction of new pollution. So-called 'clean' technologies represent the logical end-point of this discussion, when the production processes themselves assist in the reduction of waste and the minimisation of pollution, in the ultimate integrated system.

All industrialised countries face the same three problems in attempting to marry economic growth with environmental responsibility, namely the need to marshal material resources, deal rationally with their waste and the requirement for adequate and affordable energy. This dichotomy of desire between compromising neither

commercial success nor environmental stewardship is particularly important for the long-term future of the economy. Over the years, a certain brand of extremist environmentalist thought has sought to demonise industry and commerce, decrying them and casting them in the role of enemy. This is scarcely helpful, for two reasons. Firstly, if any particular industry is actively damaging the environment, it is hardly likely to react constructively to criticism from its avowed detractors. Secondly, and perhaps much more importantly, industry in its widest sense is what has defined humanity from the outset. It accounts for what our Neolithic ancestors did, trading skins and flint axes across Europe; it is absurd to suggest that our collective future will be different. The way ahead, then, is to accept this and chart a course which, if it cannot do the most good in absolute terms, must settle for doing the least harm. In much the same way as some have vilified industry, there are those who have held the idea of a self-sustaining civilisation up to ridicule, arguing that ultimately this would have us living in mud huts, devoid of all the benefits of science and technology. The one view is as facile as the other.

The issue of sustainability has gained ever greater significance over recent years, and this seems set to continue in the future. In 1987, under the aegis of the World Commission on Environment and Development, the Bruntland Commission coined a definition of sustainable development. Their concept of an approach which 'meets the needs of the present without compromising the ability of future generations to meet their own needs' has received widespread international acceptance. The main aims have been further developed into social progress to address the requirements of all, effective environmental stewardship, the maintenance of high and stable economic growth and levels of employment, and the utilisation of natural resources in a prudent fashion (DETR 1999). These goals also tend to offer strong commercial benefits and as a result, businesses have not been slow to see their potential. In a survey undertaken by the management consultancy, Arthur D. Little, of some 500 environmental, health and safety and other business executives in North America and Europe, 95% believed sustainable development was 'important'. Around 80% said it had significant real business value, while 70% of the Europeans and more than 55% in the USA reported an active sustainable development approach to strategy and operations within their organisations, for reasons of perceived business advantage. In this context, increased efficiency, competitive streamlining, better public relations, work-force awareness and rising customer expectations were all cited, while the impact of technological innovation was universally recognised.

In many respects, the move towards integration is inevitable. We cannot unscrew one leg of our tripod without unbalancing the whole structure. Sustainable development inherently demands a cogent view of resource management, and this implicitly covers materials, waste and energy. It becomes impossible to consider them in isolation. If waste becomes viewed as raw-material-in-waiting, one bridge is clear. Between waste and energy, however, the current link is incineration and, although this route will always be relevant for some unwanted materials, the

situation is less than ideal. For one thing, burning denies the bridge discussed above, by allowing little or no opportunity for reclamation. If we extend this to larger environmental issues, like reducing CO_2 production and the usage of fossil fuels, biomass, and hence environmental biotechnology, comes to occupy a pivotal position in the sustainability debate.

Bioenergy

The concept of obtaining energy from biomass material was mentioned earlier, in respect of the biological waste treatment methods involving anaerobic digestion and fermentation, and represents nothing particularly novel in itself. Methane and ethanol have been long established as fuels in many parts of the world, their production and utilisation being well documented. Both of these may be described as derived fuels, biochemically obtained from the original biomass. However, to many people around the globe, the most familiar forms of biofuel are far more directly utilised, commonly via direct combustion and, increasingly, pyrolysis. Around half the world's population relies on wood or some other form of biomass to meet daily domestic needs, chiefly cooking. Estimates put the average daily consumption of such fuels at between 0.5–1.0 kg per person (Twidell and Weir 1994a). This equates to around 150 W which is an apparently high figure, but one largely explained by the typical 5% thermal efficiency of the open-fire method most commonly encountered.

The energy demands of the developed world are well known to be enormous. In the USA alone, the requirement for electricity has grown by 2.7% on average per year over the past 10 years (Perkowitz 2000). The *Executive Order on Biobased Products and Bioenergy*, August 1999, set out the goal of tripling US biomass use by 2010, which has been estimated to be worth around $15 billion of new income, while at the same time reducing carbon emissions by the equivalent of removing some 70 million cars from the road (Feinbaum 1999). The European Commission has also suggested that the EU as a whole should aim to double the current contribution made by renewable energy sources, taking it to 12%, also by 2010. Under this proposal, biomass energy was to provide an additional 90 million tonnes of oil equivalent (Mtoe) per year, raising its overall share to 137 Mtoe. Half of this would come from specifically farmed energy crops, while other biofuel forms would account for the rest.

The energy of all biofuels derives ultimately from the sun, when incident solar radiation is captured during photosynthesis, as discussed in Chapter 2. This process collects around 2×10^{21} joules of energy, or 7×10^{13} watts, each year, throughout the biosphere as a whole. During biomass combustion, as well as in various metabolic processes described elsewhere, organic carbon reacts with oxygen, releasing the energy once more, principally as heat. The residual matter itself feeds back into natural cycles for reuse. It has been calculated that a yearly total of some 2.5×10^{11} tonnes of dry matter circulates around the biosphere, in

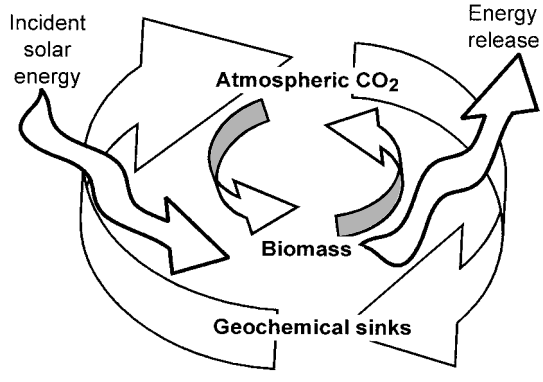

Figure 10.1 *The biomass and bioenergy cycle*

one form or another, of which around 1×10^{11} tonnes are carbon. (Twidell and Weir 1994b).

This relationship of energy and matter within the biospheric system, shown schematically in Figure 10.1, is of fundamental importance to understanding the whole question of biomass and biofuels. Before moving on to examine how integrated technologies themselves combine, it is worth remembering that the crux of this particular debate ultimately centres on issues of greenhouse gases and global warming. Increasingly the view of biomass as little more than a useful long-term carbon sink has been superseded by an understanding of the tremendous potential resource it represents as a renewable energy. Able to substitute for fossil fuels, bioenergy simply releases the carbon it took up during its own growth. Thus, only 'modern' carbon is returned, avoiding any unwanted additional atmospheric contributions of ancient carbon dioxide.

Derived Biofuels

Methane biogas

Biogas is a methane-rich gas resulting from the activities of anaerobic bacteria, responsible for the breakdown of complex organic molecules. It is combustible, with an energy value typically in the range of 21–28 MJ/m^3. The general processes of anaerobic digestion and the biochemistry of methanogenesis have been discussed in earlier sections of this book, so they will not be restated here. As mentioned previously, the main route for methane production involves acetic acid/acetate and accounts for around 75% of gas produced. The remainder is made up via methanol or carbon dioxide and hydrogen, as shown in Figure 10.2.

At various times a number of models have been put forward to aid the prediction of biogas production, ranging from the simplistic to the sophisticated.

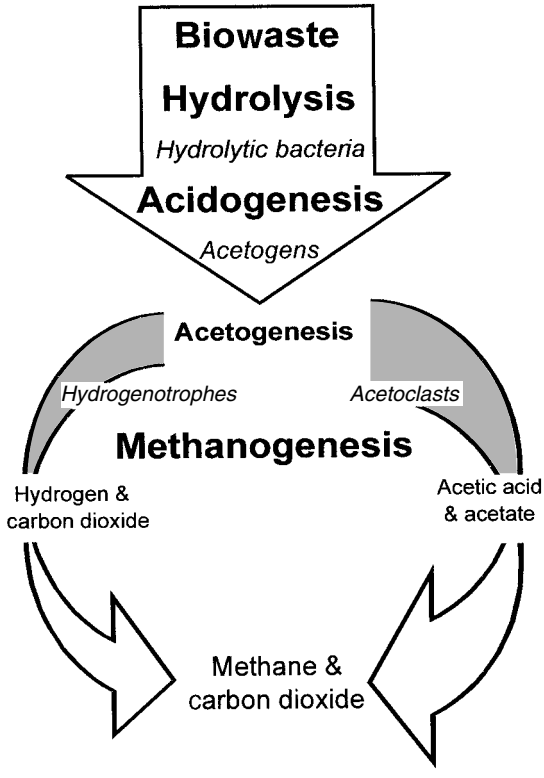

Figure 10.2 *The methanisation of biowaste*

Many of these have been based more on landfill gas (LFG) generation than truly representative anaerobic bioreactors, which does lead to some confusion at times. However, it is generally accepted that the linked, interdependent curves for cellulose decomposition and gas evolution can be broadly characterised as having five principal stages, outlined below.

- *Stage I* – Peak biowaste cellulose loadings; dissolved oxygen levels fall to zero; nitrogen, and carbon dioxide tend to atmospheric levels.
- *Stage II* – Carbon dioxide, hydrogen and free fatty acids levels peak; nitrogen levels fall to around 10%; cellulose begins to be broken down.
- *Stage III* – Carbon dioxide decreases and plateaus, to hold at around 40%; methane production commences and achieves steady state at around 60%; free fatty acids decrease to minimum levels; cellulose breakdown continues at a linear rate with respect to time; nitrogen levels fall to near zero.
- *Stage IV* – Carbon dioxide and methane continue in steady state at c. 40% and c. 60% respectively; cellulose component reduces steadily.

- *Stage V* – Cellulose becomes fully decomposed, ultimately leading to zero methane and carbon dioxide production; oxygen and nitrogen revert to atmospheric levels.

Although beyond the scope of the present discussion to address fully, the position of hydrogen as a regulator of methane production warrants a brief mention. In the earlier examination of anaerobic digestion the obligate syntrophic relationship between the hydrogen-producing acetogenic bacteria and the hydrogen-utilising methanogens, was described. Essentially, higher fatty acids and alcohols are converted to acetate, which requires an active population of hydrogenotrophic methanogens to ensure a low hydrogen partial pressure, avoiding the preferential production of butyric, lactic, proprionic and other acids instead of the desired acetic. This has the potential to cause higher volatile fatty acids to accumulate beyond the system's ability to self-buffer, leading to a lowering of the pH. In turn, as the increased acidity inhibits the methanogens themselves, methane production ceases and ultimately the process will collapse.

A number of different applications have developed the idea of anaerobic digestion for methane production, notably in the waste management, sewage treatment, agricultural and food processing industries. The process has also been successfully used at relatively small scale, commonly with animal manures as its feedstock. Figure 10.3 shows an illustrative chart of methane generation for many of the common biodegradable components of MSW.

Methane has an explosive range of 5–15% by volume and a density at 20 °C of 0.72 kg/m^3; for hydrogen the same properties lie between 4–74% and 0.09 kg/m^3 at 20 °C, respectively. At 20 °C, carbon dioxide has a density of 1.97 kg/m^3. The calorific value of typical biogas, consisting of about 60% CH_4, 40% CO_2, lies between 5.5–6.5 kWh/m^3 and it is this which makes its production attractive as a means of generating renewable energy. As was mentioned in the earlier section on anaerobic digestion, with a theoretical yield of 400 m^3 of biogas per wet cellulosic tonne, the prospect of high energy returns simultaneous with waste

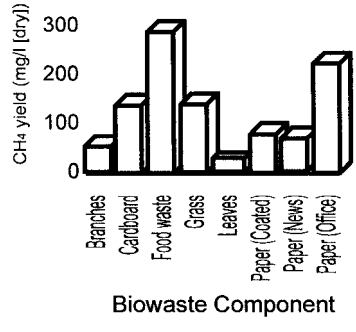

Figure 10.3 *Methane generated from biowaste components*

treatment has clear appeal. However, as was also pointed out in the same earlier segment, it is not feasible to optimise conditions such that high levels of both waste reduction and gas generation are deliverable. More commonly, in practice only around a quarter of the potential biogas yield is actually achieved.

Using biogas

Although biogas from engineered AD processes share many similarities with landfill gas (LFG), it is important to remember that it is of quite distinct quality, being much cleaner and far less contaminated by traces of other gases. LFG may contain a bewildering array of 'others', dependent on the exact nature of the waste undergoing decomposition. The list includes the likes of 1,2-dichloroethene, alkylbenzene, butylcyclohexane, carbon disulphide, propylcyclohexan, methanethiol, decane, dichlorobenzene, undecane, ethylbenzene, dodecane, trimethylbenzene, tridecane, toluene, dimethyl disulphide, nonane and sulphur dioxide. Biogas, by contrast, is relatively pure by comparison, since the bulk of the inorganic matter and many potential pollutants are excluded from the bioreactor, either by source or mechanical separation, as part of the waste preparation process. This obviates the need for high temperature flaring, commonly used for LFG to destroy residual pollutant gases, since they are highly hostile to the fabric of any generation equipment intended to be used.

The main cause for concern in this respect is hydrogen sulphide (H_2S), which is a metabolic byproduct of sulphur-reducing bacteria. Unsurprisingly, the amount present in the final gas produced depends largely on the relative abundance of sulphur-containing compounds in the original biowaste. H_2S is acidic and this poses a major corrosion risk to gas handling and electrical generation equipment. Scrubbing hydrogen sulphide out of biogas is possible, but in practice it is more common to use a high-alkalinity lubricant oil which is changed often.

Biogas utilisation involves burning it, with some of the energy being transformed to electrical. There are three basic types of engine which are suitable generating motors for biogas uses, namely turbine, dual fuel and spark ignition. For each there are numbers of different manufacturers worldwide. While, clearly, it lies far outside of the scope of this book to discuss them, it is worth noting that for any given application, the type of engine used will normally be decided by a number of contextual issues. Hence, the quantity of the biogas produced, its purity, the intended life of the plant, relevant pollution controls and other similar site-specific considerations will need to be considered.

Generation processes are generally relatively inefficient thermodynamically, and often much of the available energy is effectively lost as heat. However, the nature of engineered AD processes are such that there is a ready built-in demand for thermal energy to elevate and maintain the digester temperature. This may account for between 20–50% of the total energy produced, dependent on system specifics, in a typical temperate facility, with the remainder being available for

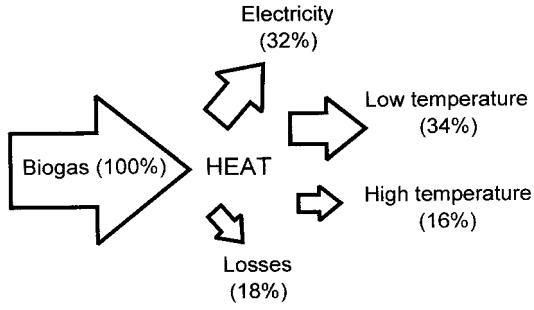

Figure 10.4 *Energy from biogas utilisation*

other uses. A representative energy flow for gas-engine generators is shown in Figure 10.4.

Ethanol fermentation

Fermentative processes have been described earlier, both in the general wider metabolic context and specifically in regard to their potential use in the treatment of biowaste. Fermentation produces a solution of ethanol in water, which can be further treated to produce fuel-grade ethanol by subsequent simple distillation, to 95% ethanol, or to the anhydrous form by azeotropic codistillation using a solvent.

The relative ease with which liquid fuels can be transported and handled, coupled with their straightforward delivery to, and inherent controllability of combustion in, engines makes them of considerable importance. Ethanol is a prime example in this respect, since it can be used either as a direct replacement for petrol, or as a co-constituent in a mix. Though at 24 G J/m^3, it has a lower calorific value than petrol (39 G J/m^3), in practice any performance discrepancy is largely offset by its better combustion properties.

There are thriving ethanol industries in many countries of the world, generally using specifically energy-farmed biomass in the form of primary crop plants, like corn in the USA and sugarcane in Brazil. In another example of the importance of local conditions, the production costs of ethanol and the market price realised by the final fuel depend on many factors external to the technology itself. Hence the indigenous economy, employment and transport costs, government policy, taxation instruments and fiscal incentives all contribute to the overall commercial viability of the operation.

Brazil, where ethanol/petrol mixing has been routine since the 1970s is an excellent example. Although the country's use of ethanol partial substitution has a relatively long history, dating back to the 1930s, the real upsurge of acceptance of 'gasohol' lay in an unusual combination of events, partly driven by the energy crisis of the mid-1970s. Rising oil prices, which increased by over 25% in less

than two years, came at the same time as a fall in sugar revenue following a slump in the world market. The Brazilian sugarcane industry, which had shortly before invested heavily in an extensive national programme of modernisation, faced collapse. Against this background, the production of fuel from the newly available biomass crop became a sound commercial move, simultaneously reducing the country's outlay on purchased energy and buoying up one of its major industries.

The keynote of this chapter is the potential for integrating biotechnologies. In the preceding discussion of biogas, this involved the marrying together of the goals of biowaste treatment and energy production. In a similar vein, as was described in an earlier chapter, there have been various attempts, over the years, to produce ethanol from various forms of waste biomass, using naturally occurring microbes, isolated enzymes and genetically modified organisms (GMOs). The appeal to obtaining renewable energy from such a cheap and readily available source, is obvious.

In many respects, the situation which exists today with biowaste is very similar to that which surrounded Brazil's sugarcane, principally in that there is an abundant supply of suitable material available. The earlier technological barriers to the fermentation of cellulose seem to have been successfully overcome. The future of ethanol-from-biowaste as an established widespread bioindustrial process will be decided, inevitably, on the long-term outcome of the first few commercial projects. It remains fairly likely, however, that the fledgling industry will depend, at least initially, on a sympathetic political agenda and a supportive financial context to succeed. While this application potentially provides a major contribution to addressing two of the largest environmental issues of our time; energy and waste, it is not the only avenue for integrated biotechnology in connection with ethanol production.

As has already been mentioned, specifically grown crops form the feedstock for most industrial fermentation processes. The distillation which the fermentate undergoes to derive the final fuel-grade alcohol gives rise to relatively large volumes of potentially polluting byproducts in the form of 'stillage'. Typically high in BOD and COD, between six and 16 litres are produced for every litre of ethanol distilled out. A variety of end-use options have been examined, with varying degrees of success, but dealing with stillage has generally proved expensive. Recently developments in anaerobic treatments have begun to offer a better approach and though the research is still at an early stage, it looks as if this may ultimately result in the double benefit of significantly reduced cost and additional biomass to energy utilisation. The combination of these technologies is itself an interesting prospect, but it opens the door for further possibilities in the future. Of these, perhaps the most appealing would be a treatment train approach with biowaste fermentation for ethanol distillation, biogas production from the stillage and a final aerobic stabilisation phase; an integrated process on a single site. There is, then, clear scope for the use of sequential, complementary approaches in this manner to derive maximum energy value from waste biomass

in a way which also permits nutrient and humus recovery. Thus, the simultaneous sustainable management of biologically active waste and the production of a significant energy contribution becomes a realistic possibility, without the need for mass-burn incineration. In many respects this represents the ultimate triumph of integration, not least because it works exactly as natures does, by unifying disparate loops into linked, cohesive cycles.

Clearly, both AD and ethanol fermentation represent engineered manipulations of natural processes, with the activities of the relevant microbes optimised and harnessed to achieve the desired end result. In that context, the role of biotechnology is obvious. What part it can play in the direct utilisation of biomass, which generates energy by a quite different route, is less immediately apparent. One of the best examples, however, once again relates to biological waste treatment technologies, in this instance integrated with short rotation coppicing (SRC).

Short rotation coppicing

Short rotation coppicing differs from simple tree husbandry, being more akin to an alternative crop grown under intensive arable production. Typically using specially bred, fast-growing varieties or hybrids, often of various *Salix* or *Populus* species, SRC involves establishing plantations which are then harvested on a sustainable basis, to provide a long-term source of biomass material for combustion. There is often a substantial land requirement associated with SRC and routinely a 2–4 year lead-in period. Once established, however, a yield of between 8–20 dry tonnes per hectare per year can reasonably be expected, with a calorific value of around 15 000 MJ/tonne. Harvesting the crop forms a rotational cycle, as different sections of the plantation reach harvestable size, year on year. In this form of energy cropping, the trees themselves are effectively pruned, rather than felled, regrowth ensuring a continuing supply. Utilisation is by burning, usually in the form of chips or short lengths, most commonly for heating purposes in one form or another. In addition, the potential for producing electricity is becoming increasingly important.

The practicalities and limitations of generation from such a fuel source largely lie beyond the scope of the present work to examine. In general, though, ensuring continuity of supply and adequate production can be problematic. In addition, while much interest has been shown in the idea of using the biomass produced by a number of individual growers in a single generator, the logistics and transport costs are major obstacles to overcome. It is possible to characterise any given fuel in terms of its calorific value per unit mass, which is referred to as its *energy density* (ED). Clearly, high ED confers obvious advantages in terms of storage and delivery. Wood, however, is a relatively low energy density fuel and hauling it to a centralised facility, thus, becomes costly, both in economic and environmental terms, especially over long distances. There is a clear advantage, then, in maximising the final yield of energy cropped trees and integrated biotechnology can assist in this regard.

The climate of the growing location, the irrigation needs of the particular trees being grown, the available nutrients in the soil and the management regime all play major deciding roles in the ultimate delivered biomass energy to land area ratio. While the climate must be simply accepted, the last three production variables can be optimised by judicious interventions.

The irrigation requirements of SRC have been the subject of much debate and consternation over the years. In this respect, some confusion has crept in between the needs of poplars and willows. While the former has a very deep tap root and in close planting can lower the water table by up to 10 times its grassland level, the latter has a much shallower root system, making no greater a demand than a normal crop like winter wheat or sugar beet (MacPherson 1995). Even so, at the equivalent of conventional arable requirements there still remains a large irrigation need and it is obvious that for locations with soils of poor water-holding capacity, this could form a major constraint, however well suited they might otherwise be for biomass production.

Integrating biowaste products

The potential for nutrient and humus recycling from biowaste back into the soil, via composted, digested or otherwise biologically treated material was mentioned in Chapter 8. Without digressing into detailed examination of the general options open for the utilisation of such soil amendments, they do have water-holding applications and form another example of the natural potential for environmental biotechnologies to self-integrate.

Much of the evidence for this has come from the field, with research conducted throughout the UK highlighting the major water-holding benefits to be gained by large-scale use of biowaste compost. It has been shown that at an application rate of around 250 tonnes of composted material per hectare, the land is able to hold between 1000 and 2500 tonnes of rainwater (Butterworth 1999). Perhaps the most significant evidence in this respect comes from the trials of large-scale compost treatment in the loose, sandy soils of East Anglia, which seem to suggest that this would allow SRC crops to be grown without any further watering in all but the most exceptional of years (Butterworth 1999). According to the same study, even under such circumstances, the additional irrigation required would be very greatly reduced. The same work established that relatively immature composts are particularly effective in this respect, as they can absorb and retain between two and 10 times their own weight of water. The situation appears similar for dewatered AD digestate, when applied to soil and permitted to mature *in situ*. Digestate sludges are often aerobically stabilised in a process sometimes rather inaccurately termed ‘secondary composting’; this approach simply extends the same idea. The end result of this process is a high humus material, with good microbiological activity and excellent water-retaining properties, which appears to match the performance of ‘true’ composts at similar application levels. Moreover, it would also seem that biologically derived soil amendment materials like

these, applied appropriately to soils either as a surface mulch or ploughed-in, can not only lower supplementary watering demands enormously but also largely offset any tendency to drought stress in the growing biomass. In addition, the leaching of nitrate from the soil is also lessened significantly.

As an aside, it is interesting to note that this ability to retain large amounts of water, together with its naturally high organic content has led to the use of compost in the construction of artificial wetlands. The USA has been particularly active in this area, in part due to the fact that federal environmental regulations encourage the creation of this type of habitat as a means of water treatment. This approach, which has been discussed more fully in an earlier chapter, has as its main goal the manufacture of a wetland which behaves like a natural system in terms of both its hydrology and biology. To achieve this, a humus rich, biologically active medium, which closely replicates the normal physical and chemical properties of local soils is required. Biowaste-derived composts have been found to contribute well as constituents of manufactured wetland soils, often allowing vegetation to become established on such sites more quickly than usual (Alexander 1999).

Nutrient requirements

To return to the issue of minerals, one of the chief potential bulk end uses of biowaste-derived compost is as a horticultural amendment and fertiliser replacement. There is no clear consensus between those working in the field as to how much nutrient is removed from the system when SRC wood is harvested, estimates for nitrogen loss ranging between 30 kg and 150 kg per hectare. A study by the UK's Forestry Commission produced figures of 135 kg per hectare for nitrogen and 16 kg of phosphate, which is around one-fifth the demands made by a cereal crop. On this basis, it seems unlikely that nutrient removal would be a limit on fertile sites and certainly not for the first few harvest cycles. In the case of soils with naturally low fertility, or those which have been used for coppice cropping for some years, supplementary mineral input may well be required. Clearly, if biowaste-derived material is used for its water-holding properties, the concomitant humus and mineral donation would represent what might be described as a gratuitous benefit. Process integration in this fashion brings evident economic advantages to any commercial coppicing operation.

There is another way in which composts can help SRC. Direct competition from other plants is one of the largest factors in poor coppice crop growth and may even lead to outright failure in some cases. Uncontrolled grass or weed growth around the trees in their first season can reduce the dry matter yield by a fifth and halve their overall growth. Even after they have become properly established, weed control remains an important part of optimising the energy crop's performance, particularly where a soil's intrinsic water-holding and/or nutrient levels are less than ideal. Heavy mulching has been used very successfully in many operations and, as is obvious from the previous discussions, biowaste soil amendments are

ideal candidates for use in this role. It is clear that the benefits of weed suppression as a means of maximising the harvested energy yield will also apply to many other biomass crops.

Agricultural benefits of compost

In general terms, it is possible to summarise the agricultural benefits of compost as the addition of humus material and nutrients, which improve soil structure and fertility, respectively. Compost brings with it a readymade microbial community which can significantly augment the complement already present in naturally impoverished soils. With better physical structure, aeration is improved and root growth facilitated. The ability of biowaste-derived material to contribute to a soil-nutrient replacement programme, and thereby lead to a reduction in proprietary chemical fertiliser use, has been a consistent finding in numerous studies. This also represents a further prospective contribution on two relevant sides of the intervention triangle. Firstly, in reducing nitrogenous inputs, it may play a useful part in reducing the farm's pollution potential. Secondly, it becomes an example of cleaner production, since by biocycling nutrients back into the chain of biomass utility, it forms a closed loop system in respect of both minerals and energy. There may still be further 'clean' benefits to come, since research at the University of Kassel on a range of plants, including cabbage, carrots, potatoes and tomatoes has found that the use of compost was associated with an improved nitrate to vitamin C ratio in the final product. Moreover, in structurally deficient soils especially, compost appears to produce better results than it is possible for artificial fertilisers alone to achieve. Even so, most investigations have concluded that while high application rates generally tend to give relatively big increases in crop yield, at lower levels the effect is less significant, being very largely attributable to the compost's humus enhancing effect.

Biodiesel

Returning to the central consideration of bioenergy, it would be wrong to discuss this topic without at least some passing reference to biodiesel, even though, since it revolves around a chemical refining process, it is not strictly produced by biotechnology. Like the increasing number of mineral oil substitutes currently available or under development, biodiesel is derived from vegetable oils. Modern diesel engines demand a clean-burning fuel of uniform quality which can function under all expected operating conditions. One of the main advantages of biodiesel is that it can be used directly, in unmodified engines, with the additional bonus that it can perform as a single, pure fuel, or as part of a mix with its traditional counterpart, in any ratio desired. While there remains some disagreement as to the scale of the environmental benefits to be gained, especially in respect of carbon dioxide discharges, there is good evidence that particulate emissions are significantly reduced. In addition, biodiesel is claimed to have better lubricant properties

and to improve the biodegradability of the conventional diesel component of a blended fuel. Various studies have concluded that biodiesel exhaust is generally less harmful to both human health and the planet. Specifically, it contains significantly lower levels of polycyclic aromatic hydrocarbons (PAHs) and nitrited polycyclic aromatic hydrocarbons (nPAHs), which is of great importance, since both groups have been identified as potential carcinogens. In laboratory tests, PAHs were reduced by between 75–85 % (excepting benzo(a)anthracene for which the figure was around 50%) and nPAHs were also dramatically lessened. Most of the targeted nPAH compounds were present only as traces, while the highest levels reported, 2-nitrofluorene and 1-nitropyrene, were found to represent a 90% reduction over typical conventional diesel releases. Objective views of the performance of a 'new' fuel depend on such information and the National Biodiesel Board was congratulated by representatives of the House Energy and Power subcommittee for being the first industry to complete the rigorous health effects testing of the Clean Air Act.

It is not entirely without irony that in 1894, when Rudolf Diesel invented the engine which bears his name, he produced a design specifically suitable for a range of fuels, including coal dust and vegetable oil, as well as the petroleum product which is automatically associated with the device. In many respects, the current resurgence of interest in the potential of a fuel source so deeply rooted in diesel's origins might almost be described as a retrograde step in the right direction.

External regional considerations

As stated at the outset, biodiesel is a product derived by the application of chemistry to material of biological origin and is not, thus, biotechnological in the truest sense. It does, however, illustrate very well the influence of local modality as one of the recurrent themes of the sector. For a time, use of biodiesel in the USA was confined to certain niche markets, principally because relatively low conventional fuel prices and the contemporarily high cost of vegetable oil made wider uptake unattractive. However, the impact of the legislative requirement for alternative fuel in the Energy Policy Act of 1992 (EPACT) has led to a major upsurge in usage, especially amongst bus operators and hauliers, for whom it is the most cost-effective option available.

In Europe, by contrast, the economic and environmental benefits may be less clear cut. The UK's Royal Commission on Environmental Pollution's major report, *Energy and the Changing Climate*, published in June 2000, largely ignored biodiesel, concentrating its attention more on farmed energy crops for use in combined heat and power stations. Of biomass crops for vehicle fuel, it was much less enthusiastic. Its examination of European Union funded research on oilseed rape as a raw material for biodiesel led it to identify quality control issues and conclude that the actual production of biodiesel is polluting, and is inefficient in terms of both energy and cost. The economics aspect is a major one.

According to official figures from the Department for the Environment, Food and Rural Affairs (DEFRA), in 2001, UK rape seed sells for 15% less than it costs to produce, even after taking government subsidies into account (Curran 2001). February 2001, eight months after the Royal Commission's report, saw the European Commission publish the first review of its 1997 strategy for renewables, entitled *The Communication on the Community Strategy and Action Plan on Renewable Energy Sources*. In this document, the poor adoption of liquid biofuels like biodiesel, was specifically criticised, with only Austria, France, Germany and Italy having defined policies on usage. Even so, their combined contribution to the total diesel fuelled transport sector only amounted to 0.3% in 1998, the latest period for which figures were available. As is so often the case, the report concluded that revised taxation to favour biofuels will be the key to future expansion, coupled with the establishment of specific objectives and greater incentives for the growing of energy crops under the common agricultural policy. These were largely adopted by November of the same year, when biofuels were prioritised in the EU as part of a strategy to reduce petroleum product dependency for transport. At the present rate, burgeoning European oil imports are predicted to increase to 90% by 2030, if no steps are taken. The first phase of a planned 20% substitution by 2020 will involve legislative and fiscal promotion of these fuels, which are to account for 2% of all fuel sold by 2004.

The carbon sink or energy crop question

As a final and more general environmental point on this topic, as was mentioned earlier, the realisation has been growing that using biomass in a balanced way, combining its undoubted value as a carbon sink with a progressive substitution for fossil fuels, has certain clear advantages over the sequestration-only option. Energy crop production is based on a sustainable cycle which brings benefits to the soil as well as both local biodiversity and the local economy. Land bound up in carbon sinks does not offer appreciable employment; energy-farmed biomass crops can support jobs, both directly and indirectly within the region, which has evident importance for rural diversification, itself a major countryside issue.

Integrated Agricultural Applications

The farming industry is almost certainly about to change dramatically and the importance of novel production crops of the future will not, it seems, be limited to the energy sector. As Senator Tom Harkin of the Senate Agriculture Committee pointed out in June 2001, the potential exists for anything which can be made from a barrel of oil to be manufactured from farmed produce of one kind or another. The realisation of this is growing on a global basis and it is, therefore, highly likely that a considerable part of the forthcoming development of agricultural biotechnology will move in this direction. For reasons which should be

obvious, and follow on logically from much of the preceding discussion, there is a natural fit between agricultural and environmental biotechnologies and hence, a significant potential for integration both between and within them.

Some of the ways in which this can take place in respect of biowaste-derived soil amendment products have already been described and, clearly, the advantages they convey are not limited to the particular energy crop examples cited. Before leaving this particular topic, there is another aspect of their application which is worthy of note, not least since it illustrates both integrated production and a potential means of obviating current dependence on a significant environmental pollutant.

Plant disease suppression

Intensively reared crops can suffer extensive and expensive losses resulting from plant disease infection. Until the early 1930s, crop rotation and the use of animal manures and green mulches provided the traditional protection regime; after this time, chemical fumigation became the favoured method to deal with soil-borne pathogens, which can accumulate heavily in intensive monocultures. Methyl bromide has been the main agent used, its popularity largely attributable to its ability also to destroy weeds and resident insect pests. It is, however, an indiscriminate tool, and though it has contributed directly to the commercial viability of many growers' operations, it has been implicated in ozone depletion. Accordingly, under the terms of the Montreal Protocol, it is due to be phased out by 2005. This, coupled with other fears regarding residual bromine in food and groundwater, has led to bans in Germany, Switzerland and the Netherlands, the latter being, at one time, Europe's largest user of methyl bromide soil fumigation.

A number of alternative options are being explored, including soil pasteurisation using steam, ultraviolet treatment and the development of resistant cultivars using both selective breeding and genetic modification. The use of compost extracts – so-called 'compost teas' – is also receiving serious consideration as a means of crop-specific disease control. Their action appears to be two-fold, firstly as a protection against foliar diseases and secondly as a inoculant to restoring or enhancing suboptimal soil microbial communities.

Research projects in Germany, Israel, Japan, the UK, the USA and elsewhere have found that these extracts are very effective natural methods to suppress or control a number of plant diseases thus reducing the demand for artificial agrochemical intervention (Table 10.1). Direct competition with the relevant pathogen itself is one of a variety of mechanisms believed to play a part in the overall disease suppression, along with induced disease resistance, and the inhibited germination of spores. This is thought to be brought about by means of the extract's action on the surface of the leaves themselves and stimulatory effect on the associated circum-phyllospheric micro-organisms. Bacteria, yeasts and fungi present in the extracts have been shown to be active agents, while evidence points to a number of organic chemicals, including phenols and various amino acids, also

Table 10.1 *Plant disease suppression using selected compost extracts*

Compost extract	Suppressed disease
Bark compost extract[1]	*Fusarium oxysporum* Fusarium Wilt
Cattle compost extract[2,3]	*Botrytis cinerea* Grey Mould of beans and strawberries
Horse compost extract[2]	*Phytopthora infestans* Potato Blight
Manure–straw compost extract[4]	*Plasmopara viticola* Downy Mildew of grapes Sphaerotheca fuliginea Powdery Mildew of Cucumbers Uncinula necator Powdery Mildew of grapes
Spent mushroom compost extract[5]	*Venturia conidia* Apple Scab

[1] Kai, Ueda and Sakaguchi 1990.
[2] Weltzein 1990.
[3] Elad and Shtienberg 1994.
[4] Weltzein 1989.
[5] Cronin *et al.* 1996.

having a role in the suppression effect. The exact nature of this action is not fully understood, but it would seem to be principally a biological control, since it has been known for some time that fine filtration and sterilisation by heat treatment, significantly reduce the extract's effectiveness (Trankner 1992).

Compost teas are prepared for use by either aerated or fermented extraction methods. So-called 'fermented' extraction was the original, first developed in Germany and it is not, in fact, a fermentative process at all. Actually an infusion method, this requires a suspension of compost in water to be made, in a ratio typically around 1:6 by volume. The resultant mixture is allowed to stand for a given period, usually between 3–7 days, then coarsely filtered prior to being used. The second method, which came out of research in Austria and the USA, is more active and, with a typical cycle period of around 10–12 hours, derives the product in much shorter time. The acceleration is achieved by increasing the oxygen transfer to the extract during formation, initially by passing water through compost, collecting the resultant liquor and recirculating it many times to concentrate and aerate. Once prepared by either method, the finished product is used as a foliar drench, typically applied to commercial crops at a rate of around 1000 litres per hectare (100 gallons per acre).

The abilities of properly prepared biowaste composts themselves to suppress and control soil-borne plant diseases, especially where mature compost is directly mixed with the soil itself have been established (Serra-Wittling, Houot and Labouvette 1996). The efficacy of the protection given by this kind of incorporation with soil known to be conducive to plant pathogens has also been demonstrated.

In particular, the deleterious effects of various species of Phythium, *Phytophthora* and *Fusarium* as well as *Rhizoctonia solani*, which is a major threat to many kinds of young plants, are largely suppressed or controlled. For many years the horticultural industry had been aware, anecdotally and from experience, that composted tree bark suppressed root rots caused by *Phytophthora* spp. Investigations of this revealed that plants which require the presence of vasicular arbuscular mycorrhizae (VAM), small fungi around the roots, which are intimately involved in nutrient and water uptake, grow better in blends of soil and bark compost than in methyl bromide treated soil alone. Various bacteria, fungi and yeast, largely indigenous to the original compost, have subsequently been indicated as active agents in the overall effect. The biological nature of the control has been established in laboratory trials involving heat treatment or microwave exposure of the compost. This significantly altered the microbial balance, resulting in progressive inhibition of suppression and led to 100% plant mortality in some instances. On this basis, the need to ensure that compost is not subjected to extreme temperatures during storage or transport becomes a very obvious problem to be avoided in any intended practical application.

The agricultural usage of biowaste-derived products has considerable potential, but public acceptability and quality assurance issues must be major concerns, probably more here than in any other comparable sector. In the UK alone, farmers have not been slow to learn the terrible consequences of consumer anxiety. Bovine spongiform encephalopathy (BSE) and the passions raised by genetically modified (GM) crops, or animals reared on them, entering the human food chain has focused attention ever more clearly on supply chain issues. The ramifications, in both economic and social terms, of the foot and mouth disease (FMD) outbreak, which paralysed the UK farming industry throughout 2001, has left the rural community all too well aware of the meaning of biosecurity. Since today's agri-business is so largely dominated by the demands of the supermarkets, it is neither unreasonable nor unlikely that guaranteed product quality would be a requirement in any industry-wide standard. A clear precedent has already been set in this respect with the involvement of the British Retail Consortium in the evolution of the matrix safety code for the treatment and application of sewage sludge to agricultural land. The drive towards so-called 'organic' farming has already fostered a climate of proliferating, and typically widely differing, compost acceptability criteria throughout the world. This is scarcely helpful to the average would-be user of these products, often serving more to confuse than elucidate.

The prospect of widespread uptake in the growth of bioproduction crops in the future has been suggested as one of the routes forward for agriculture. To echo Senator Harkin's words, farmed resources could well account for much of what is currently derived from crude oil, either directly in the chemical sense as an alternative source of the same product, or indirectly as substitutes. In the final analysis, the acceptance of the latter will, inevitably, depend on factors which are more societal and economic than scientific or technical and as such,

largely beyond the scope of this book to discuss. Suffice it to say that, in any novel application, cost is a major issue and although the potential market may be enormous, the commercial benefits must be clear. As discussed before, the attitude of industry will be crucial. There is undoubtedly a strong background interest in bioproducts, but machinery is often extremely expensive, and down-time is costly and inconvenient. Using a bioengineered substitute which has not been tested and approved, often represents a huge commercial gamble, and it is a risk which few enterprises, understandably, can afford to take. It may be some time before the oft-quoted image of vast areas of transgenic crop plants growing the biological equivalents of today's petroleum-based products, at no more cost than cabbages or corn, finally becomes a commercial reality. However, the beginnings are clear.

The search for a biological method of producing plastics, for example, already shows some promise. The ability of some bacteria to produce natural polymers has been known for some time and a number of attempts at growing plastics have been made. The products typically proved expensive, costing between three and five times as much as ordinary plastic and were generally found to be too brittle for normal use. Poly(hydroxyalkanoates) are a class of natural polymers with thermoplastic properties, which can be synthesised by bacteria, though the process is itself economically uncompetitive. Using green plants as plastics factories promises much greater competitiveness, but guaranteeing the appropriate monomer composition is not easy. One solution was recently demonstrated using genetically modified varieties of oilseed rape and cress, engineered to produce poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) a Poly(hydroxyalkanoate) with commercial applicability, within their leaves and seeds (Slater *et al*. 1999). In effect, this is a perfect example of genetic engineering manipulating the respective strengths of contributory organisms to optimise their functional isolated potential. Inserting the bacterial genes responsible for plastic production into *Arabidopsis* and *Brassica* plants, avoids the expense of feed, since photosynthesis naturally provides the necessary carbon and the metabolic flow of intermediates from fatty acid and amino acid synthesis is redirected for plastic production. The PHBV biosynthesised in plant plastids appears to be of a marketable type and quality, though the yield was relatively low. The process will need some refining if it is to be a realistic proposition for serious commercial production.

Clearly, if and when it is, returning to the intervention triangle model to consider the agricultural benefits of specifically *environmental* biotechnology, it will represent a major advance in both 'clean' production and pollution control. While the former may largely remain for the future, in many ways the latter is already achievable.

Microbial pesticides

Chemical pesticides are problematic for many reasons. Firstly, although some degradation occurs as described in Chapter 4, pesticides are notoriously

recalcitrant and consequently their use may lead to a build-up of chemicals damaging to the environment. This is an increasing problem with the intensive drive towards more and more cost-effective crop production. Secondly, insects are known to develop resistance to pesticides and so new and in some ways, more poisonous chemicals might be introduced to maintain the same level of effectiveness. Thirdly, chemical pesticides are rarely targeted to specific problematic species and may kill other organisms of no harm or even of some benefit to the crop plants. Balanced natural environments have an equilibrium between assailant and victim, however, it may take a commercially unacceptable time for this balance to establish, sometimes incurring quite extreme swings in either direction. For example, one season may see a flourishing of citrus trees due to an outbreak of disease leading to a dearth of butterflies, the caterpillars of which feed voraciously on citrus. The lack of insect host reduces the level of infection, leading to a recovery of the butterfly population the following year and, consequently, seriously damaged citrus trees. One means by which insect numbers are controlled in nature is by bacteria which produce toxins killing the insect which consumes it. Although this may serve to create the balance described above, it may not be sufficient to satisfy commercial crop production. Sufficient time may elapse between ingestion of the toxin by the larva and its ensuing death, for it to have caused considerable damage by feeding on the crop. Perhaps the best studied pesticidal bacterial toxin is the δ-endotoxin from *Bacillus thuringiensis*. This protein, frequently abbreviated to 'Bt toxin', is active against some members of the Lepidoptera (butterflies and moths), Diptera (flies, midges and mosquitoes) and Coleoptera (beetles) families and has been used in its native, unmodified form as a pesticide for many years. There are several strains of the bacterium each one producing a toxin active against a limited number of insect species; a relationship which is continuously evolving. Already successful, it would appear to be the current leading candidate for development into a more generally useful and effective biopesticide, thus hopefully reducing the dependence on chemical pesticides.

There are limitations associated with its use, all of which are being addressed in active research. These include a limited range of insects susceptible to each toxin, requiring dosing with multiple toxins, insufficient ingestion by the insect to prove lethal in a usefully short time, stability of the toxin when sprayed on crops and the development of resistance by the insect. The last stumbling block has attracted particular interest (Roush 1994, Gould 1994, Bohorova *et al*. 2001). The genes coding for the toxins have been isolated opening the way to their alteration and introduction into suitable 'delivery systems', either bacterial or into the plant itself, offering the plant inbuilt protection, thus attempting to overcome the various limitations introduced above.

However, even without genetic engineering, *Bacillus thuringiensis* in its native form, remains a widely used and successful product for commercial crop protection especially in 'organic' farming. In practice, the only major developments

found to be necessary, are improvements to the physical formulation of the crop spray; dry, flowable formulations being an advance on the original wettable powders. Increasingly, nematodes especially *Steinernema* sp., are demonstrating great potential as biological control agents applied as sprays. It is anticipated that they will complement *Bacillus thuringiensis* in extending the spectrum of pests controlled by 'environmentally friendly' means (Knight, R., Koppert Biological Systems, personal communication).

There are other *Bacillus* species which have also been used effectively as microbial insecticides. These are *Bacillus sphaericus* which produces a toxin more potent but more specific than Bt, and *Bacillus popillae* which although it does not produce a toxin, kills its host by weight of bacterial numbers. The latter is active against Japanese beetle, while the former is quite specific against mosquito larvae. Both the mosquito larvae and *Bacillus sphaericus* abound in heavily polluted water such as cesspits where the bacterium may exert control on the proliferation of mosquitoes. A different approach to microbial pesticides has been to examine the exploitation of Baculoviruses. The drive to use Baculoviruses as a means of biological pest control has dominated its research in the past, but currently these viruses are being recognised more as vectors to express proteins of various origins at a very high level indeed and so have become enormously useful tools in the major branches of biotechnology. Several Baculoviruses are registered for use in the USA as insecticides against maize bollworm (*Heliothis zea* SNPV), gypsy moth (*Lymantira dispar* MNPV) Douglas fir tussock moth (*Orgyia pseudotsugata* MNPV) and in the UK against pine sawfly (*Neodiprion sertifer* MNPV) and pine beauty moth (*Panolis flammea* MNPV). Members of the Baculovirus family, also called Nuclear Polyhedrosis Virus for reasons which become clear with a knowledge of their replication cycle, have been isolated from Lepidoptera, Homoptera (aphids and their relatives) and Diptera. The infectious cycle passes through a stage where several virus particles are bound together in a large crystal of protein. This protects the virus particles until the crystal is ingested by an insect where enzymes in the gut digest this polyhedrin protein releasing the viruses. These enter the insect's cells where they are uncoated, make their way into the cell nucleus, and the viral replication cycle commences. Some 12 hours after initial infection, virus particles are released which spread the infection to neighbouring cells. By 24 hours post infection, the protective polyhedrin protein coded for in the viral DNA, is being produced in sufficient quantities to start assembling the crystal structures. By this time all the insect tissues are suffering severe damage such that at the time of death, the insect is effectively a mass of virus particles surrounded by the insect's cuticle. This cadaver is eaten by birds, and consequently may be spread some distance. The virus is carried intact in the bird's gut protected in the polyhedra, which is resistant to digestion by the enzymes found in avian gut. Polyhedrin protein is made in enormous quantities in the infected cell, but since its only known function is to protect the virus *in vivo*, in the wild, then it is redundant when culturing the

virus *in vitro*, in the laboratory. This being the case, the coding sequence for the polyhedrin protein may be replaced by a 'foreign' gene where under the control of the polyhedrin promoter it may, depending on the gene being replaced, have a good chance of being expressed at a very high level. This methodology used to overexpress proteins, which was described in Chapter 9, has been used with great success but increasingly in biotechnology fields other than environmental, notably pharmaceuticals.

Plant/microbe interactions

The microbiology of soils and plant microbe interactions are enormous topics worthy of the many books and research papers on the subjects, some of which are listed in the bibliography. It is not the aim of this section to give a detailed account but simply an introduction to the complexity of plant–microbe interactions in the hope of illustrating that 'no man is an island': disturbance of these interactions has its consequences. Although the term 'plant' includes all plant forms from trees to algae, the current discussion addresses interactions between higher plants and micro-organisms. Such interactions fall into two basic categories: the first category being those involving microbes external to the plant, such as soil bacteria and soil fungi. The second being microbes internal to the plant, which include endophytic bacteria such as those involved in nitrogen fixation, internal fungi, and plant pathogens examples of which are *Agrobacterium plasmodium* and *Agrobacterium tumefaciens* (Greene and Zambryski 1993). The latter is now used extensively to introduce 'foreign' genes into plants as described in Chapter 9. The associations may therefore involve bacteria, fungi or viruses and in some cases, some quite complex interactions involving three or four different organisms often bringing great benefit to the plant in environments where nutrients are somewhat deficient.

Microbes external to the plant

There are clearly two distinct areas of a higher plant which are inhabited by different communities of micro-organisms: above ground around and on the surface of leaves, stems, seeds and flowers and below ground in zones of increasing distance away from the root mass. These rhizospheres, or zones around the roots, which are more accurately envisaged as a continuous gradient of nutrients, are the result of plant metabolic activity constantly drawing from the surrounding soil. Nutrients may also be transferred in the reverse direction that is, to the soil, as is the case with aerating plants exemplified by *Phragmites* used in reed bed systems described in Chapter 7. It appears that colonisation of the rhizosphere by bacteria is stimulated by exudate from the plant. The first phase is attraction to the plant roots, the second is a 'settlement' phase during which bacteria grow to form colonies and the third is a 'residence' phase when a balance is established between root mass and bacterial numbers (Espinosa-Urgel, Kolter and Ramos

2002). The microbes in the rhizosphere are, to a large extent, dependent on the plants for a supply of many useable organic substances. As plants die and decay, the components released by the degradative bacteria return to the soil and the cycle begins again. Consequently, plants affect the composition of the microbial community of the ground in which they grow, especially in soils of low fertility. Not all the compounds released from plants are stimulants of microbial growth, some plants may also produce inhibitors. The microbes themselves have an effect on the plant growth characteristics. Some release into the soil, gibberellins and cytokinins, both of which are plant growth factors, and may also affect the flow of organic compounds, termed exudate, from the plant into the rhizosphere. The rate at which exudate is transferred to the soil is affected by many parameters; the presence of surrounding soil bacteria as mentioned above, the reduction of plant mass by harvesting from above ground level, and environmental changes, for example, variations in light or temperature. Both bacteria and fungi contribute to the microbial population in the rhizosphere. Associations of fungi with roots of vascular plants, called mycorrhizae, are quite common and may in some cases be very beneficial to the plant. They may be external, ectomycorrhizal, or internal, endomycorrhizal. Ectomycorrhizal associations more commonly occur in temperate regions and often in beech, oak, birch and coniferous trees. Their association involves a limited penetration of the root cortex by the fungi growing as a covering around the tip of the root. They aid the growth of the plants as a result of their mycelia reaching far out into the surrounding soil, thus assisting the plant in nutrient uptake. This quality has received commercial attention. The effect on plant growth and subsequent predation by insect larvae, of some species of fungus for example, *Pisolithus tinctorius*, has received particular attention (Rieske 2001). Bacteria have been found capable of encouraging this association, earning themselves the title of 'micorrhizal helper bacteria'. Clearly, anything which increases the efficiency of nutrient uptake by crop plants reduces the requirement for the addition of artificial fertilisers and thus reduces the potential for agrichemical environmental disturbance.

The influence of microbes on the welfare of plants is not confined to the ground and may even affect the weather. An often quoted example is that drawn from *Pseudomonas syringae* which produces a protein known to act as a point of nucleation of ice crystals. Plants which harbour this bacterium run an increased risk of frost damage especially if their tissue is particularly susceptible as is the case with strawberries. *P. syringae* has been subjected to genetic engineering which successfully reduced the problem. A description of the project is given in Chapter 9.

Plant–microbe interactions are becoming recognised as having an immediate and direct importance to human health in the role they can play in reducing the effect of 'sick buildings'. They occur principally because these buildings are closed systems in which people work, breathing in volatile components from plastics, paint, chemicals used in office machinery such as photocopiers and

printers and a range of other sources including furnishing manufactured using synthetic materials. Bacteria resident in the soil of potted plants in the office are able to degrade many of these volatiles, which include phenolics, formaldehyde and trichloroethylene, thus improving the air quality. The plants themselves contribute to this improvement, not only by supporting the rhizosphere microbial community, but also by producing oxygen during photosynthesis described in Chapter 2. In essence, this is root zone phytotechnology on a small scale.

Although straying away slightly from the remit of this section, it is interesting to point out that soil microbial activity has a major influence in the balance of stable atmospheric gases. These include the greenhouse gases, carbon dioxide, nitrous and nitric oxide and methane, so called because they trap heat re-emitted by earth from energy radiated by the sun. The atmospheric balance of less stable gases which include ammonia, hydrogen sulphide and dimethylsulphide are also subject to microbial activity, as will be apparent from an understanding of the foregoing chapters on metabolism. A final word on soil microbes concerns the degradation of lignin. This is a major constituent of woody plant material and is recalcitrant to degradation. However, filamentous fungi are responsible for its degradation worldwide, augmented in the tropics by bacteria living in the gut of termites. This degradation requires the presence of oxygen, hence wood residing in anaerobic conditions is somewhat protected. Clearly, should the water table drop, exposing to air the wood pilings supporting buildings, for example, there is a danger of invasion by filamentous fungi able to degrade lignin and thus weaken the building structure. This also explains in part the necessity to aerate a compost heap containing any woody plant material to allow invasion by filamentous fungi capable of degrading lignin.

Microbes internal to the plant

Two categories fulfil this description. The first are the internal fungi or endomycorrhizae, referred to in the earlier section, together with the endophytic bacteria and the second comprises plant pathogens, which may be bacterial or viral in form. Although the term ‘endophytic’ seems unambiguous, it is sometimes used to describe only bacteria which may be isolated from plants which have been superficially cleansed with disinfectant, or isolated from within plant tissue and which cause no discernible harm to the plant. Thus defined, plant pathogens are excluded from this description. There are endophytic bacteria, called commensals, which neither benefit nor harm the plant, but there are also those which are beneficial to plant growth. These are symbiots which achieve this status either by promoting plant growth or by protection against plant pathogens.

Symbiotic nitrogen fixation

The classic example of plant growth stimulation by plant/microbe symbiosis is nitrogen fixation by *Rhizobium* bacteria within plant root tissue. A full exposition of nitrogen fixation may be found in many classical textbooks and so

will be described here only in outline. The process of root nodule formation is shown diagrammatically in Figure 10.5. In total, there is a limited number of organisms able to fix nitrogen, all of which are prokaryotes. All living organisms are dependent ultimately on such organisms, due to a universal and essential requirement for nitrogen, normally in the form of nitrate, ammonia or ammonium ion or as amino acids from which the amino group may be transferred as required. Nitrogen is fixed by reduction to ammonia either by free living organisms or by plant symbiots. In both cases, it is essential to have an oxygen free environment as the enzymes involved in the process are irreversibly inactivated by the presence of oxygen. Of the free living organisms able to fix nitrogen, some achieve this naturally while others have to create such an environment. *Clostridium* and *Klebsiella* achieve this since they are both anaerobes and so are already adapted to life in an oxygen-free atmosphere, while *Cyanobacteria* and *Azobacter* have developed means of creating one for themselves. *Azobacter* does this by having a very high oxygen consumption rate thus effectively creating an oxygen-free environment. Other nitrogen-fixing bacteria include the filamentous bacteria such as the *Corynebacterium* species, and photosynthetic bacteria referred to elsewhere, such as *Rhodospirillum.* The latter makes use of photosynthesis to provide the energy for these reactions and so is presented with the problem of removing the oxygen produced during photosynthesis away from nitrogen fixation reactions. Although these free living organisms have a vital role to play in their particular niches, approximately 10 times more nitrogen is fixed by plant symbiots. Presumably this is because the plant is better able to provide the necessary levels of ATP to meet the high energy demands of the process than are free living bacteria. In addition, the plant supplies the endophytes with dicarboxylic acids, such as malate and succinate and other nutrients, like iron, sulphur and molybdenum which is a component of the nitrogen-fixing enzymes. It also provides its nitrogen-fixing symbiots with an oxygen-free environment, as described later in this discussion. The analogous chemical process has an enormous energy requirement to achieve the necessary high temperature, in the region of 500 °C, and a pressure in excess of 200 atmospheres. This is part of the reason why manufacture of fertilisers for agricultural purposes, which is in effect the industrial equivalent of the biological process, is a drain on natural resources. Together with the unwelcome leaching of surplus fertiliser into waterways, causing algal blooms among other disturbances, this makes the widespread application of fertiliser recognised as a potential source of environmental damage. It is understandable to see a drive towards the engineering of plants both to increase the efficiency of nitrogen fixation, which is estimated at being 80% efficient, and to extend the range of varieties, and especially crop species, which have this capability. It is also worth noting that unlike superficially applied fertilisers which may exceed locally the nitrogen requirement and so leach into the surrounding waterways, nitrogen fixation by bacteria occurs only in response to local need and so is very unlikely to be a source of pollution.

The following brief description of the required interactions between plants and microbes, the example used here being *Rhizobium*, should serve to illustrate why such a goal is very difficult to achieve. Firstly, the plant is invaded by a member of the *Rhizobium* family of free living soil bacteria. There is a specific relationship between plant and bacterium such that only plants susceptible to that particular member of the group may be infected. Genes involved in the infection process and nodule formation called *nod* genes are coded for by the bacterium. The *nod* genes are activated by a mixture of flavenoids released by the plant into the region around the roots and thus the plant signals to the bacterium its receptiveness to be infected.

After infection through the root hairs, the multiplying bacteria find their way into the cells of the inner root cortex. They are drawn into the cell by endocytosis shown in Figure 10.5 and so are present within the cell bounded by

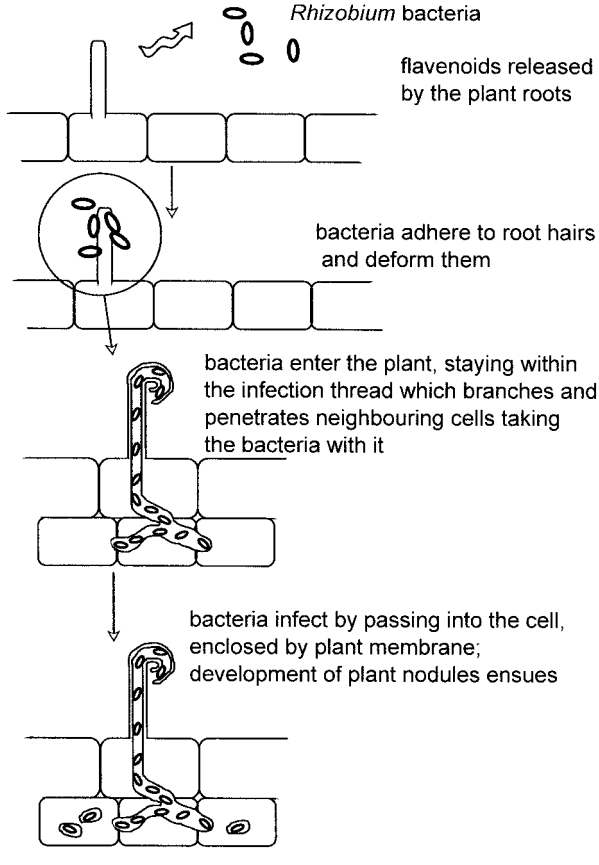
Rhizobium bacteria
flavenoids released by the plant roots
bacteria adhere to root hairs and deform them
bacteria enter the plant, staying within the infection thread which branches and penetrates neighbouring cells taking the bacteria with it
bacteria infect by passing into the cell, enclosed by plant membrane; development of plant nodules ensues

Figure 10.5 *Root nodule formation*

plant cell membrane. This structure then develops into nodules containing the nitrogen-fixing bacteria. Several changes to the plant then ensue including the synthesis of proteins associated with the nodule, the most abundant of which is leghaemoglobin, which may reach levels of up to 30% of the total nodule protein. The genes coding for this protein are partly bacterial and partly plant in origin and so exemplify the close symbiosis between the two organisms. The expression of leghaemoglobin is essential for nitrogen fixation, since it is responsible for the control of oxygen levels. The enzymes for fixation are coded for by a plasmid of *Rhizobium* and are referred to as the *nif* gene cluster. There are two components each comprising a number of genes. One component is *nitrogenase reductase* the function of which is to assimilate the reducing power used by the second component, *nitrogenase*, to reduce nitrogen to ammonium ion. Expression of the *nif* genes is highly regulated in all organisms studied to date. In addition to the *nif* and the *nod* genes, *Rhizobium* also carries additional genes which are involved in the fixation of nitrogen called the *fix* genes. Once ammonia, or the ammonium ion, has been formed it may be transferred to the amino acid, glutamate, to produce glutamine. This transfer is frequently invoked throughout metabolism. This is not the only route for the assimilation of the amino group into metabolic pathways; the synthesis of the purine derivative, allantoin or allantoic acid, being a less heavily used alternative. These pathways are described in Chapter 2. Nitrogen fixation involving *Rhizobium* has been the focus of the preceding discussion, but the family of aerobic, Gram positive bacteria, Actinomycetes, is another group carrying out the same function using a similar mechanism. These may form a network of aerial or substrate hyphae resembling a structure commonly associated with that of fungi. These bacteria may form a close association, called actinorhizae, with the roots of a number of plants which tend to be woody, or shrub-like in nature.

Endomycorrhizae

It is not only bacteria which may live within plants, but some types of specialised fungi may also occupy such a niche. Their presence is fairly widespread and may be found in various grasses and a wide range of commercial crop plants including tomatoes, apples, beans, wheat and corn. One type of plant–fungus association, actinorrhizae, has already been mentioned in the section describing symbiotic nitrogen fixation. The fungal hyphae penetrate the plant cells where a variety of structures may develop such as swellings or the development of coils or small branches. Vesicles and arbuscules which are branched structures reminiscent of a tree, are common features of this invasion. Despite how this description may appear, such association of plant with fungus may be very beneficial to the plant. In exchange for energy derived from the plant through photosynthesis, the fungus may enhance the supply of available nutrients to the plant under conditions of relatively high humidity and conversely, in dry conditions, the fungus may help the plant in the uptake of water. In addition, some fungi have been found to afford

protection to the plant in cases where the fungi produce alkaloids rendering the plant less susceptible to attack by chewing insects.

Plant pathogens

There are many bacteria, fungi and viruses which may infect a plant and cause disease, fungi being one of the major causes of plant disease. Most micro-organisms will not be pathogens for a particular plant as the manifestation of disease requires some quite specific reactions and responses between host and infectious agent. Infection elicits numerous responses some of which may be quite complicated, and so has been the centre of some fairly intensive research. The result of this has been the identification and isolation of plant genes involved in resistance to pathogens and pathogen virulence genes. Manipulation of these to reduce the level of environmentally damaging chemicals to protect crop plants is an active area of research and the manipulation of these genes is discussed in the previous chapter. This section is restricted to two examples, one bacterium and one virus, which are chosen because of their relevance to plant genetic engineering described in Chapter 9. The bacterium is *Agrobacterium tumefaciens* and the virus is Cauliflower Mosaic Virus.

Agrobacterium tumefaciens

A feature of infection caused by this Gram positive organism, is a tumour-like growth, seen as a crown gall in plants. This is the consequence of injection by the bacterium of a small piece of DNA which carries on it genes which code for opines which encourage further invasion of the plant by the bacterium, and for plant growth hormones including auxin whose activity stimulates plant growth thus producing the characteristic tumour. It is interesting to note this example of gene transfer from prokaryote to eukaryote occurring with moderate frequency in nature. The significance of this is explored further in Chapter 11. The genes coding the information required for the insertion of this small piece of DNA including the insertion sequence itself, are carried on a plasmid, described in Chapter 2, called Ti plasmid whose structure is shown in Figure 9.4. The process of infection is stimulated by exudate from a plant which has been wounded by some means not necessarily the result of infection, for example, by frost damage. The *vir* genes are activated leading to nicks being introduced at the borders of the T-DNA leading to the release of one of the strands of the double-stranded DNA. A copy is made of the remaining strand by the usual methods of DNA synthesis and repair thus restoring the status quo of the Ti plasmid. The single-stranded piece of T-DNA is free to be transferred into the plant cell through a wound site and on into the plant cell nucleus. Here the complementary strand is synthesised in the normal way using the plant cell enzymes and the resulting double-stranded DNA integrates into the plant genome. The T-DNA comprises three genes including those for plant hormones and opines, as mentioned above.

Once the T-DNA is integrated into the host plant cell genome, these genes may be expressed leading to the establishment of crown gall disease. *Agrobacterium tumefaciens* then contains a very effective delivery system for bacterial genes into a plant cell; a natural process which is utilised by genetic engineers to introduce 'foreign' genes into plants as described in Chapter 9.

Cauliflower mosaic virus

The study of plant viruses has lagged behind that of animal or bacterial viruses due to difficulties in their culture and isolation. While some may be grown in isolated plant cultures, many require the whole plant and some also require an insect intermediate either simply as a means of transmission between plants, or additionally as a site for virus replication. Transfer of viruses between plants by insects is the most common means, especially by insects which penetrate and suck plants, aphids being an example. However, there are other routes such as transfer by plant parts through infected seed, tubers or pollen. Other agents are those able to penetrate plant tissue which include soil nematodes and parasitic fungi. The genetic material of most plant viruses is RNA, either double or single stranded. Caulimovirus, or more commonly, Cauliflower Mosaic Virus is unusual in having a DNA genome. This has proven very fortuitous for genetic engineering as it possesses two very strong constitutive promoters, the 35S and the 19S; for an explanation of the term 'promoter' see Chapter 2. Since these promoters originated from a plant virus, any construct made with the intention of expressing the 'foreign' gene in a plant has a higher likelihood of the signals being recognised by the transcription machinery of the plant than if they were derived from, for example, bacterial promoters. These promoters have proved very successful and particularly the 35S, often designated in publications as '35S CaMV', has become almost the archetype promoter to drive the expression of 'foreign' genes in plants. Examples of these are given in Chapter 9. However, while genetic engineering, building on these and other natural abilities, may permit novel technological approaches to emerge, which have major potential relevance to the environmental sphere, currently they are of somewhat limited commercial application. At present, there seems to be much more scope, at least in practical terms, for the bundling of existing technologies into treatment trains, or the re-entry of post-processed biological material into the chain of commercial utility.

Closing Remarks

Biotechnological integration often permits a number of key environmental concerns to benefit. The use of phytotechnology using whole organism systems to clean effluents has been discussed in an earlier chapter. Recently, waste biomass from the brewing industry, which normally represents a disposal problem to the industry, was found to have unexpected abilities to remove a number of common

metal pollutants from effluents. Something in the region of 100 000 tonnes of hops are grown yearly worldwide, but only the cones are used, leaving the rest as waste. When mashed, mixed into a silica-based polymer and then formed into granules, the hop residual biomass was discovered to be as effective at removing contaminating cadmium, chromium, copper, lead and zinc ions from wastewater as conventional commercial ion-exchange resins. Moreover, preliminary evidence suggests that the hop-residue system specifically targets certain metals, avoiding the problems that resin-based exchangers typically have with 'hard water' ions like magnesium and calcium. It is a common criticism of environmental biotechnology that all it ever achieves is to move a problem from one place to another and there would be something in this if the story ended here, leaving the question of the ultimate disposal of the metal/biomass combination unresolved. However, using a mild acid treatment, over 90% of the metals collected can be recovered, and the plant material remains suitable for reuse. Although commercial application of this emergent technology is still some way away, it does illustrate the potential power of integration to tackle multiple environmental concerns, in this case addressing issues of waste, effluent and recycling.

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Case Study 10.1 Integrated Pollution Control (Hoy, Orkney)

While there are many examples available of the use of biotechnology to mitigate environmental contamination, perhaps one of the most interesting illustrations lies in the integration of pollution control between a controlled internal environment and the outside world.

Fish farming has often been the target of much criticism for its effect on local waters, typically being blamed for pollution and reduced natural biodiversity or indicted as a reservoir of disease to the detriment of wild populations. Leaving aside the fact that many of these accusations are simply unfounded, the industry itself has a clear vested interest in the sustainable use of water. Accordingly, many fish farmers have developed approaches to their business which reflect a very practical concern for environmental protection. The Rackwick Hatchery on the island of Hoy, Orkney, typifies the sort of best practice which can be achieved by even relatively modest operations.

Water recycles through the growing tanks, returning to a central sump from which it passes into a biofiltration system designed by the proprietor, John Eccles, to maximise treatment efficiency while minimising energy use. As it trickles down through the filter elements, nitrates, nitrites and ammonia are removed and the water is re-oxygenated beyond the requirements of the resident biofilm microbes, thereby partially reconditioning it for reuse in the fish-growing units. The arrangement of the water return, which deliberately encourages surface splashing, continues this process with a net result that the fish farm has a significantly reduced requirement for abstracted fresh water.

Independent monitoring of the adjacent Rackwick burn by the Scottish Environmental Protection Agency (SEPA) has established that there is virtually no difference in water quality between upstream and downstream of the facility, the small variance noted, lying well below the relevant acceptable limits. By integrating good husbandry, conventional UV sanitisation techniques and effective bioengineering, the fish farm has managed to meet a number of environmental goals, achieving low resources demand in terms of energy and water, together with effective pollution control, without compromising the internal conditions necessary for commercial production of high-quality fish protein.

Case Study 10.2 Practical Bioenergy (Canada and the USA)

Attempts to derive energy from waste biomass have often been criticised over the years, typically being cast as incineration by another name. Forestry and wood wastes in particular, lend themselves to true 'thermal recycling', but this can only be a justifiable environmental option when wider considerations are taken into account.

An example of this is provided by the advances made by Talbott's Heating, Stafford UK, in meeting stringent pollution control targets on the international market. Having been manufacturing and exporting systems for some 30 years, the company has seen considerable changes in regulation and continuous research and development has enabled them to meet all necessary requirements. In addition to the unique

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proprietary design, the biomass combustion units are typically installed with monitoring equipment to ensure compliance with the limit set for particulates, carbon monoxide, temperature or other emission parameters, dependent on local requirements.

The company now has over 3500 working installations in 20 countries of the world and recently set up business in Vernon, Canada, to service growing demand throughout North America. Although the emissions standards demanded are amongst the toughest in the world, it has proved possible to meet them without compromising operational efficiency. The system provided for Spinks Lumber of New York State is a good illustration. Despite stringent environmental legislation, the company's wood waste, which has a typical moisture content around 60%, provides process hot water for kiln drying and heating, even though temperatures can drop to −40 °C at certain times of the year.

The principal market for biomass fired heating systems currently lies within the wood working industry, where the dual benefits of cost-effective waste disposal and 'free heating' have been long recognised. The future may not be so restricted as developments in emissions control increase the number of materials which can be safely burnt. Thus, certain kinds of treated timbers, cardboard, paper and even some types of plastics are now just as suitable as virgin wood, which may have some interesting implications for approaches to waste management in the coming years. In the light of the system at Princeton Canada to heat commercial greenhouses, the possibility also beckons of near-total carbon recycling. Initially driven by the spiralling cost of meeting their huge energy needs with gas, the installation produces hot water from palletised wood. Suitable quality CO_2 is subsequently produced from the flue gases generated, and returned to enrich the local atmosphere inside the greenhouses, to benefit plant growth. While this is not a closed system, nor entirely carbon neutral in the local sense, it does begin to illustrate some of the future potential of this approach to energy.

11
The Way Ahead

Implicit in the very essence of biotechnology is the idea of the use of biological systems for commercial benefit and consequently, as has been a recurrent theme throughout much of this book, this inescapably means that it is subject to business pressures. Economic, legislative and political forces will shape the future of environmental biotechnology every bit as much as they have its past. Such is the inherent nature of the subject. At the same time, the use of biological solutions in environmental applications depends on the current state of the art and this is ever changing as new developments are made. One of the areas where major advancement may occur is in the field of biosensors.

Biosensors

Some are currently available which have been very successful in environmental monitoring of pollution. A genetically modified yeast has been developed which detects oestrogen, 17ß-oestradiol and other molecules many of which are also endocrine disrupters. Although this sensor was designed for use in human therapeutics, there is the potential for use in pollutant detection, especially in the light of concern over these chemicals in the watercourses, although considerable development is required (Tucker and Fields 2001). However, the use of biosensors for 'one-off' analyses such as in testing materials about to be tipped into landfill sites, must await refinement to a much higher level of reliability. The possibility of producing them with enormous sensitivity and selectivity is becoming a reality with the development of 'microchip' type sensors combining biological activity with nanowire electronics (Cui *et al*. 2001a). This technology has reached a stage of being able to detect a change in an electric current attributable to the binding of a single molecule (Cui *et al*. 2001b). Although these studies raise exciting prospects for biosensors, the 'ever-present' question of cost remains for environmental applications.

Manufacturing

In the manufacturing sphere, it seems certain that there will be increasing pressures to continue the reductions in pollution and waste production. These are

related issues, of course, and both may be addressed by one of two general approaches: *a priori* methods to avoid the problem in the first place or *a posteriori* to clean it up more efficiently after the event. Clean production methods, which have been variously discussed in Chapters 4 and 10, seem likely to play a growing part in achieving the former. The use of various production biotechnologies will limit the environmental impact of ever more industrial processes by reducing their energy demands, the strength of effluents generated, the amount of waste requiring ultimate disposal and so on. The growing importance of biocatalysis, and the general use of biological macromolecules in manufacturing procedures will, inevitably, lead to the development of novel and innovative techniques for many substances currently generated by conventional means. There are many candidates for the forthcoming major roles in the bioindustrial production cycles of the near future but probably amongst the most revolutionary and beneficial are likely to be found amid the ranks of the extremophiles, which were previously discussed in Chapter 3. Extremozymes isolated from these bacteria and archaea, which dwell happily in some of the most unlikely and biologically challenging of environments on the planet, offer the potential to catalyse reactions previously the exclusive realm of physical chemistry. Their potential lies both in primary action and secondary effect. The first of these utilises enzymes obtained to bring about the desired effect, where such specificity of action is a natural characteristic of the donor microbe. Secondary effects arise by virtue of the elucidation of the functionally key features of naturally occurring substances. This allows the same mechanisms to be incorporated into artificial chemicals which subsequently achieve a goal for which no direct analogue exists in nature. In this respect, for instance, further study of extremozymes isolated from hyperthermophilic organisms may well permit the mechanism of their heat tolerance to be discerned and the appropriate means incorporated into other, non-natural catalysts.

The value of the extremozyme contribution has already been noted in the now well-established PCR technique, which enabled a major jump ahead to be made in the whole science of biotechnology itself. In addition, the commercial use of an enzyme obtained from another thermophile to increase the efficiency of cyclodextrin production from cornstarch is a currently known example of clean technology. Though these compounds are valued in the pharmaceutical and food industries, where they principally aid the stabilization of volatile ingredients, the process can still fairly be said to have an environmental component. Improved manufacturing efficiencies typically go hand in hand with reduced pollution, waste production or energy demand. In the future, this aspect of industrial activity seems set to assume far greater importance and extremophile research may well provide many of the necessary tools to make it possible.

Thus, hyperthermophilic extremozymes have potential applications in many industries, offering amylases for confectionary or alcohol production, proteases for amino acid production, baking, brewing and detergents, xylanases for paper

bleaching, and dehydrogenases and oxidoreductases for a variety of commercial uses.

In the chemical industry, the possible use of whole-organism hyperthermophiles offers new ways to produce hydrogen, methane and hydrogen sulphide. At temperatures between 18–80 °C and under anaerobic conditions, this latter gas, for example can be made by *Desulfuromonas* from elemental sulphur. The conventional chemical catalysis system requires a temperature of 500 °C or more to yield the same result. Unsurprisingly then, the potential of whole-cell microbial biocatalytic methods and their notably superior specificity, is viewed with great interest. In future, it may be possible to redesign the configuration of conventional bioreactors to produce efficient, high temperature substitutes for many of the currently standard industrial processes.

Other extremophiles could also have roles to play. Psychrophiles may yield enzymes which will function at the low refrigerator temperatures typically required to avoid spoilage in food processing, for enhanced cold-wash 'biological' washing powders and in perfume manufacture, reducing evaporative fragrance losses. A use has been suggested for halophile enzymes in increasing the amount of crude oil extracted from wells, though whether this will ever be a commercial reality remains to be seen and, moreover, leaves aside any consideration of the 'environmental' aspects of increased fossil fuel extraction. Acidophilic extremozymes may one day form catalysts in chemical syntheses in acid solution, and alkaliphile derived proteases and lipases may replace existing versions in washing detergents to enhance their action. In addition, some of the textile industry's enzyme-using processes may see alkaliphilic extremozymes replacements for greater efficiency.

However exciting the prospects of extremophile use may be, turning their potential into industrially deliverable processes will not be straightforward. For one thing, many of these organisms are found in very specific and specialised ecological niches and replicating their optimum environmental requirements is likely to prove difficult, particularly within bioreactor systems initially designed around mesophile cultivation. Hence, new types of reactors and, possibly, novel solid-state fermentation techniques may need to be developed before this can be achieved. Commercial-scale cultures of extremophiles for extremozyme harvesting is also likely to prove problematic, since by virtue of their habitats, it is rare to find large numbers of any given single species in nature. For such purposes, the isolation and purification of the required microbial culture to be grown up is generally both difficult and costly to do. Though the extremozymes can be produced using recombinant DNA methods, as were discussed in Chapter 9, avoiding the need for wholesale, mass culturing of the extremophiles themselves, any industrial attempt at whole organism biocatalysis will, of course, demand it.

Despite the clear biotechnological potential of extremophile clean manufacturing, a complete comparison between these emergent technologies and conventional methods will inevitably be required before they are likely to gain

mainstream industrial acceptance. There is no doubt that they have advantages, but issues like cost and guaranteed reliability will prove vital in their uptake.

Clean manufacturing as a field appears to have a major contribution to make to the environmental cause and there are a number of possible novel manufacturing biotechnologies emerging. Particularly when implemented alongside 'green' chemical processes, they promise significant advances in pollution reduction over the coming years.

Waste Management

The second of the intervention points, waste management, is also likely to see major changes in the future, which are almost certain to be driven by external pressures to restrict the entry of biologically active material into the disposal loop. With strict diversionary and recycling targets increasingly becoming the accepted norm in many countries of the world, it seems certain that biowaste will be subject to expressly biological treatment at a previously unprecedented rate. It appears reasonable to assume that this will lead to an upsurge in the amount of waste destined for existing biooptions like anaerobic digestion and composting rather than any burgeoning evolution of new treatment technologies, particularly in the case of domestic waste. In addition, if clean manufacturing techniques based on biological systems become established, the amount of biodegradable waste produced in the first place should fall, though this does not allow for the contribution of normal refuse production growth, which seems to average around 5% increase per year.

The role of integrated technologies in dealing with waste while simultaneously allowing components, either material or energy, to re-enter the chain of commercial utility is likely to prove a vital one. In whatever form this most fundamental of recycling is achieved, an adequate final market is essential. Whether the end product is fundamentally reclaimed humus and minerals or a useable fuel, without a genuine and environmentally beneficial end-use of some kind, the whole operation becomes little more than an exercise in shifting the problem from one place, to another. This is a criticism which has been levelled at environmental biotechnology at various times over the years and, on occasions, with some justification. A rational use for biomass energy is unlikely to be hard to find; a mass market for vast amounts of compost, particularly if of questionable quality, may not be so easy. How the ideals of carbon sequestration are best translated into reality is still an area of some debate and will probably remain so well into the foreseeable future.

One of the great advantages of waste biotechnologies, especially when applied to domestic refuse, is the ease with which they can be incorporated into treatment trains. Indeed, biological waste treatment naturally lends itself to many applications which enable very effective maximisation of overall process efficiency to be achieved, which is an important aspect to be considered in the planning of

any intervention or structured response. Local authorities and, increasingly, commercial operations, are commonly faced with the need to make hard decisions regarding their waste management provision. The inherent flexibility and intrinsic pollution mitigation offered by engineered biotreatment, as opposed to disposal and consequent incidental biodegradation, remains a major influential factor in this respect.

Pollution Control

The future of pollution control and minimisation is, clearly, highly interdependent on the forthcoming activities within the preceding two intervention zones. With cleaner production techniques in the offing and more comprehensive biological treatment of the waste produced, not to mention the economic, legal and public relations constraints operative, *de novo* contamination should be very largely reduced. The clean-up of historically polluted sites seems likely to become a growth area for a number of reasons. The economic balance between bioremediation and other forms of removal or disposal appears to be shifting on a global perspective. In addition, the spiralling demand for commercial and housing land in many parts of the world seems set to create compelling incentives for the reclamation and redevelopment of old sites.

In this respect phytoremediation is a technology likely to see much greater development in the future, since its initial uses have been largely successful, though generally limited in scale. There are clearly many advantages to the use of whole organisms which can be managed using existing husbandry methods and the presence of which on a site is almost universally welcomed. However, not all of the approaches available to biotechnology are so well received.

Genetically Manipulated Organisms

There is a great deal of alarm felt by the general public with regard to the safety of genetically manipulated organisms (GMOs) being released into the environment, especially as the knowledge is not available to state categorically exactly the risk involved in the horizontal spread of the 'foreign' genes. There is much active research addressing the spread of introduced genes, for example, in the use of chloroplasts as targets for 'foreign' DNA rather than the plant genome. This, and various other issues raised by the use of recombinant DNA technology in environmental biotechnology, are addressed in a recent review (Daniell 1999). These may be approached at many levels with regard to environmental biotechnology, as opposed to biotechnology in general.

An obvious area for the future involvement of GMOs, or their products, would be in the development of biosensors like the example given earlier. Their use is

likely to be confined to the laboratory or 'field' test kits, and so are unlikely to warrant concern with regard to creating their own environmental pollution.

It has already been noted that GMOs are unlikely to occupy a significant niche in bioremediation, principally due to the high cost in developing such organisms set against the low financial return on remediation. Where they are likely to be of great benefit is in solving problems for which no cheaper or simpler alternative technology is available; examples of these are not widespread.

However, genetic engineering does, and will continue to, find a role in clean technology as in examples quoted in Chapter 10. There is great potential to develop 'designer biocatalysts', either as isolated enzymes, or as whole cells, which should go a long way to helping industry improve its profitability and environmental profile (Burton, Cowan and Woodley 2002). The picture is also somewhat different where environmental biotechnology strays into agribiotechnology. Here the potential for the development of genetically modified plants with improved quality or increased resistance to harsh conditions or pathogens, as described in Chapters 9 and 10, is apparent.

Concerns about the safety of such constructs should be viewed in the light of discussions in Chapter 3 emphasising the natural mobility of genetic material. In nature, there is a considerable amount of genetic exchange even between unrelated organisms. A powerful example is that of the insertion of the Ti plasmid of the bacterium, *Agrobacterium tumefaciens,* into plant cells and another is the 'mariner' gene shown to have 'jumped ' from tsetse fly to human (Edwards 2000). A criticism frequently levied at genetic engineering is that it crosses species boundaries, thereby transferring genes into organisms which could never be recipients. An appreciation of the potential degree of genetic rearrangement and exchange through the activities of plasmids, viruses and transposable elements leads to the conclusion that genetic engineering can be viewed as a very tiny part of the overall picture. Furthermore, considering that there is an argument suggesting a blurring between genomic and extrachromosomal genes, it seems unrealistic to describe any gene as truly 'foreign' when the genome is probably carrying an array of genetic material originating from a variety of sources. Generally, public alarm comes with the widespread release of large numbers of specially engineered organisms into the environment, carrying genes which may be envisaged causing an environmental problem greater than the one they seek to solve. Such examples would be GM plants carrying genes for increased herbicide or pesticide resistance.

In Chapters 9 and 10, reference was made to baculoviruses being used as insecticides. There are many reasons why this appears to be a relatively safe agent given that these nuclear polyhedrosis viruses (NPVs) are unable to infect plants, microorganisms, vertebrates, or nonarthropod invertebrates. Assuming that this argument is not invalidated by alteration to the host range, recombinant baculoviruses should be even safer, since normally the protective polyhedrin protein gene has been sacrificed in favour of the 'foreign' gene. This lack of

polyhedrin protein would be expected to lessen NPV survival in the wild and certainly reduce their infectivity thus making them poor vectors for the spread of 'foreign' genes.

Another area of major concern is that that many of the constructs released into the environment carry genes for antibiotic resistance. In Chapter 9, the rationale for including such genes was described but, briefly, its function is during the construction of the recombinant and, especially in eukaryotic recombinants, serves no function in the final GMO. This being the case, it can be argued that it is reasonable to require the removal of all such selector and reporter genes before release of the GMO. Accordingly, it is especially true because of the limited number of selector and reporter genes in current use. Consequently, it is likely that while the gene or genes of interest may be unique, or almost so, to that construct, it will probably be carrying one of a very limited number of selector or reporter genes. As a result, the total number of GMOs released worldwide in one year, carrying one particular selector or reporter gene could be very high indeed. Given that true figures for the rate of gene transfer between unrelated organisms (horizontal transfer) have not yet been satisfactorily estimated, it would seem prudent to err on the side of caution and remove all unnecessary genetic material, prior to GMO release.

Attempts to estimate the rate of gene transfer in the environment are being made using microcosms. These are small-scale reproductions of an enclosed test environment. One such experiment investigated the effect of simulated lightning on the rate of plasmid transfer between bacteria. Their results showed an increase in transformation suggesting that, under certain conditions, lightning is able to make bacterial cells competent to receive plasmid DNA by horizontal transfer (Demaneche 2001). Microcosms are useful test systems especially as in this example, where the question being asked is very specific, but they have their limitations in the assessment of wider horizontal gene transfer, due to the difficulties in recreating the natural environment. It seems likely that many questions regarding the spread of genes will not be answered until sufficient GMOs have been released and the ensuing results monitored. It is clear that much more research is required into the balance between real benefits and risks of genetically engineered plants (Wolfenbarger and Phifer 2000).

Closing Remarks

In the final analysis, life is enormously robust and resilient, not perhaps at an individual level, but certainly on a gross scale. Living things, and most especially microbes, have colonised a truly extensive range of habitats across the planet, and some of these are, as has been discussed, extremely challenging places. This, combined with the lengthy history of bacteria and archaea, which has equipped many species with an amazing array of residual metabolic tools, adds up to a remarkable reservoir of capabilities which may be of use to the environmental

biotechnologist. Humanity recently celebrated the turning of the millennium – the passage of 40 generations of our species. On this basis, a bacterial 'millennium' passes in less than a day. There is a tendency, particularly in older biology textbooks, to describe bacteria and their kin as 'primitive life forms'. While, of course, this is true in terms of relative organisational complexity, it can encourage a sort of unwarranted phylum-ist view of our own evolutionary superiority. With 40 generations passing in under 24 hours and predating our own earliest beginnings by several hundreds of millions of years, prokaryotes are clearly far more highly evolved than ourselves or any other form of life on earth. Unsurprisingly, then, many of the environmental problems encountered today have readily available solutions which make use of the natural cycles, pathways and abilities of entirely unaltered micro-organisms.

As this book has been at pains to point out, while there may well be a role for the use of GMOs in some applications, it seems unlikely that engineered organisms will assume centre-stage in the field. Part of the reason is that there may simply be no need; there are enough odd abilities around quite naturally. Scientists from the US Geological Survey recently announced the discovery of hydrogenotrophic archaean methanogens living 200 metres below the surface of Lidy Hot Springs, Idaho (Chapelle *et al*. 2002). This microbial community is unlike any previously discovered; normally arachea seldom comprise more than 1–2%, but here, in conditions of low organic carbon content (around 0.27 mg/l) but significant levels of molecular hydrogen, they amount to around 99% of the population. Under normal circumstances these archaeans, being less efficient energetically, are out-competed by 'normal' carbon-eating microbes, but in the absence of solar energy, which drives ecosystems based on utilising organic carbon, conditions heavily favour them.

This has been seen as particularly relevant to the search for extraterrestrial life, since finding living systems on earth in environments analogous to those believed to exist on Mars or the Jovan moon, Europa, could provide vital clues. In many ways, perhaps it has even more relevance as a testimony to the extraordinary biodiversity of this planet and to the enormous potential of its collective gene pool.

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